

Russian science still needs more help

Mr George Soros, the financier-philanthropist, has created a mechanism for helping Russian science to survive that should be used (or at least copied) by others, public and private.

EARLY next month, there will be a meeting in Moscow of the board of the International Science Foundation, better known as the Soros fund. The business will be largely that of approving decisions already made about the allocation of funds to researchers in Russia and other former Soviet republics, but no doubt there will also be gloomy ruminations about the more distant future. In just over a year, the fund will have spent or committed most of the US\$100 million made available at the end of 1992 by Mr George Soros, the Hungarian-born financier who has devoted much of his substantial gains on the US stock market and such places to the cultivation of what he calls the Open Society in Central Europe (including even the Balkans). Although Soros has offered to match with his own money future contributions to his fund for the support of research in Russia and the other republics, the chances that his initiative will be able to continue on a sufficient scale are not, at this stage, bright.

That is a cruel shame. For the past eight years, the case for special support for research in Russia (in what follows, shorthand for all the former Soviet republics) has been overwhelming. It is simply put. Hamstrung though it was by bureaucracy, Russian science differed from other aspects of Soviet culture in being part of the international research enterprise. It is (or was) also an important part of it, and not only in mathematics and theoretical physics. The explanation is simple and heartening: not only was science a Leninist cause, but it was one of the durable achievements of the Soviet system to create a school curriculum that delivered general numeracy as well as literacy and which, in contrast with too many school curricula in the West, engendered enthusiasm for science. Of course, the concentration of research in institutes run by central academies (and separate from the relatively free-thinking universities) was not just a notorious cause of inefficiency, but also the means by which power brokers kept their underlings in thrall. The prospect, when *glasnost* and *perestroika* were first in the air, was that science as a whole would benefit from the liberation of this under-used pool of talent.

Response

Sadly, what was then an opportunity has come to seem more like another irksome charitable obligation, but governments in the West have been too slow to respond on either score. The most common (but not general) response has been to allow that collaborations with *in situ* Russian researchers may be supported by research grant applications to national

funding agencies. (The snag is that these procedures often require applicants in the West to favour Russian students or postdoctoral workers over more immediate colleagues.) Other public agencies have sought to keep Russian researchers at work by making research contracts with their institutes, usually with disappointing results: the contracts have been too vague to generate tangible research programmes, while the payments offered have proved to be less generous than they appeared at the outset, in the first flush of the market economy. If research were a simple commodity, purchasing it from Russian shelves would still be economical. The underlying reason why trade of this kind between Russian institutes and the West is unlikely to be fruitful is that its long-term value is what stays in researchers' heads.

Consequences

The consequences of indifference and neglect have been those predicted. The outward flow of able people from Russia has been sustained and damaging to the civilian enterprise left behind. Inevitably, the better institutes have been the most seriously affected. Yet, by all accounts, the productivity of Russian research courses has never been greater; every graduate student knows that the sooner his dissertation is complete, the sooner he can apply for a job elsewhere. Meanwhile, there are signs that the flow of young people into research is declining in Russia, as it has been in the West, in the face of the familiar and almost daily proof that other pursuits are more rewarding and no less insecure. It will be a sad end to the optimism of eight years ago if Russian science loses the special claim on public attention that it then held.

That is why, even at this late stage, institutions in the West in a position to help should try to put flesh on the bones of the good intentions they have been parading for several years. To be fair, there are some helpful developments. After two years of argument in the Russian parliament, a fund put together by the United States and European governments has been enabled (by presidential decree in Moscow) to begin spending money. The European Commission is similarly spending money, while the Royal Society (thanks to funds found by the British Office of Science and Technology) is half-way through a three-year programme of modest scale. But these public funds are too late and, partly for that reason, too little.

Private foundations, on the other hand, properly reluctant though they have been to put their funds at the mercy of the



bureaucracy, have been inconspicuous in the East. They should swallow their pride and use the Soros mechanism instead. So have been universities in the West. Yet one of the hopeful developments in Russia is the government's new determination to strengthen the capacity of the public universities for research, much along the Western model. That should be a sign to universities elsewhere, especially those in Europe, that the time has come to forge new links in the East.

What will come of all this? It would be wrong to expect too much. Governments and institutions in the West are all too ready to proclaim that they, too, have problems occasioned by the ending of the Cold War. The difference, of course, is that the problems of Russian research are at once more serious and more likely to have permanent consequences. But why bother, if the most able people have already moved West? There are two kinds of answers. First, if nothing substantial is done, the scientific enterprise as a whole will be harmed. Second, the political benefits of a more enlightened Western view of Russian science could ultimately be substantial, as Russia's weekend diplomatic coup in Bosnia should have emphasized. It will be ironical if the only substantial recognition of this need is that of a man (Soros) whose wealth derives in part from spotting when governments follow unrealistic monetary policies. □

New ways to a PhD

The British government is insufficiently radical in its proposals for revamping the PhD.

NOBODY can accuse the British government of inaction, at least where the education of the young is concerned. Fresh from the regulation of the curriculum on religious education (see *Nature* 367, 496; 1994), it is now hoping to lay down the law on entry to PhD courses, at least for students seeking public support (see page 674). To be sure, the proposals are neither unexpected — they were advertised in last year's policy statement on research — nor exceptionable. The idea that there should be a prequalifying year for intending PhD students is the norm in France, for example, where the DEA is arguably the most competitive of public examinations.

The defects of the extra year now proposed for Britain are partly that it aims to kill too many birds with one stone. Will it be feasible, even in an extended academic year of 42 weeks, to give young people a deeper knowledge of their chosen discipline, a broad knowledge of cognate disciplines and "transferable skills and knowledge" likely to be valuable in careers outside universities while engaging 60 per cent of their time on research? But there is also every likelihood that the effect of the prequalifying year for publicly supported students will be to reduce the number of people eventually graduating with a PhD. To be sure, the government now says that it will be for the research councils to decide how much of their resources to put into research training, but the demand for research grants proper will probably ensure that, with a stagnant budget, there will be no extra for students. For a country as short of high-level

skill as Britain, that is not a joke.

In the circumstances, the Office of Science and Technology (OST) could with advantage have been more adventurous. One of the anomalous features of the present British arrangements for PhD training is that some university departments are awarded a quota of research studentships which they can then fill with candidates of their own choice. OST implies, in its consultation paper, that this system will remain in place, but that the research councils will be expected to monitor a system for the assessment of students singled out for admission to PhD courses. That is only logical, but it will not avoid some obvious abuses of the present system (among which are academic incest and the engagement of research students on projects made up to keep them occupied).

Why not instead abolish the quota system? That is the preferable course, especially when the number of universities has been greatly increased (by the accession of the former polytechnics to university status)? And why not at the same time ensure that PhD students are always engaged on meaningful projects by linking public support for research students (they are paid a pittance, and their host departments a similar amount) to the award of research grants for successful projects? That way, a person with a good idea could expect not merely to be awarded research expenses, but also some of the (admittedly untrained) manpower required to carry it through.

There would be objections from the universities, of course. Studentship quotas are jealously regarded and fought over, for example. More to the point, universities may reasonably argue that it is for them, and not for the research councils, to appoint students to their rosters. But there would be no substantial difference from the present system if it were left to successful grant applicants and their heads of department to select those employed. Indeed, if there is to be a national system for the prequalification of PhD students in Britain, there should be a wider spectrum of young people from whom to choose. Then the universities' complaint that PhD students must not be over-constrained by research targets is countered by the simple observation that British universities are well used to registering research assistants financed by grants for a PhD.

Such a switch would also have positive virtues. Not least, it would enable universities without strong research departments to provide prequalification courses for their students, in the expectation that some of them would be able to embark on PhD courses elsewhere. And it would enable the research councils, without taking cosmic policy decisions, to begin paying graduate students respectably. That would be good for everybody's self-respect. And no harm would come from the increased length of time spent on winning a PhD. The British university system, in which most students earn a first degree in three years and a PhD in a further three, is now a kind of academic forcing-house. If each phase took an extra year, the remedial measures now planned would not be necessary. That would cost the government more, but so will the remedial measures. It would be better to bite that bullet now, not later. □

Fusion research to face 'make or break' demands from US Congress

Washington. Arguments over the US fusion research programme are likely to reach record temperatures in this summer's budget debates in Congress, when environmentalist groups trying to kill the programme are expected to clash with proponents arguing that it needs to expand in order to reach a critical mass.

At stake will be not only the future of the \$650-million Tokamak Physics Experiment (TPX) planned for the Princeton Plasma Physics Laboratory (PPPL) in New Jersey, but also the fate of the \$10-billion International Thermonuclear Experimental Reactor (ITER) programme, now at the engineering design stage.

Senator Bennett Johnston (Democrat, Louisiana), chairman of the appropriations subcommittee that holds the purse-strings on the Department of Energy's (DoE's) fusion research, has threatened to bring down the shutters on the whole programme unless he gets a firm commitment from the administration to the construction of ITER.

In the light of the Superconducting Super Collider fiasco last year, Johnston wants both Congress and the administration to face up squarely to the issue of funding the US share of ITER's construction. Otherwise, he says, the US domestic fusion programme — including TPX — will be a waste of time.

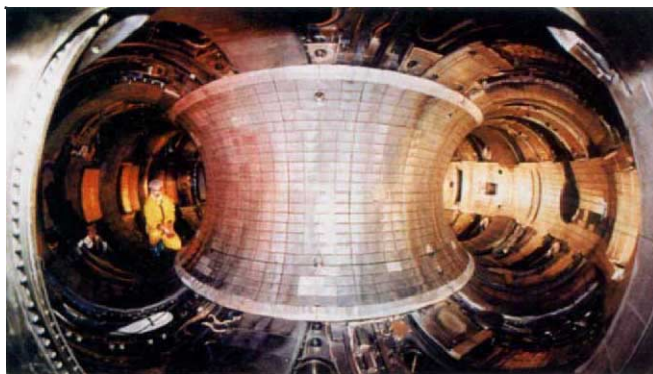
Johnston wants the administration to act earlier than is currently being planned, and formally to invite its international partners — Europe, Japan and Russia — to commit themselves to the construction phase of ITER. The White House Office of Science and Technology Policy and officials at the DoE are working on a response.

Johnston's all-or-nothing stance reflects a widespread view both in and outside Washington that the fusion programme, whose budget has fallen steadily in real terms from a peak in 1977, is now spread too thinly to achieve its ambitious goals.

Another view is that the big fusion machines such as TPX and ITER are a waste of money, and that an energy source whose proponents admit will not become commercially viable until well into the next century does not deserve the largest single share of the energy research budget.

This position is likely to gather support as environmental lobby groups, until now occupied in cutting back the US fission research programme, switch their attention to fusion.

The Clinton administration was generous to fusion in its recent budget proposals, suggesting an increase from \$344 million to \$373 million next year, while other energy programmes are being cut. The largest elements are \$77 million for research and construction of TPX, and \$69 million for ITER design work, while research facilities at San Diego and the Massachusetts Institute of Technology share \$67 million for basic research.



Inside the 2-metre-high TFTR at Princeton Plasma Physics Laboratory, due to upgrade if the money can be found.

The five-year ITER design phase is being conducted jointly on three sites — near Munich, in Tokyo and in San Diego, California — and is due for completion in 1998. The partners are expected to sign a protocol next month to confirm their commitment to the end of that phase of the process, opening the way for discussions on the thorny question of where ITER should actually be built. The engineers want that agreed by 1996.

The siting decision will not be easy. If Russia is unable to contribute, the host country is likely to have to pay an extra share which may amount to half the costs.

Some physicists fear that Europe, Japan and the United States may end up playing an embarrassing game of pass-the-parcel with this expensive proposition. The 'winner' will then have to cope with the prospect of flagging political support in the 'losing' countries.

A bill already introduced by Johnston and passed in the Senate says that, if plans for ITER falter, then the energy secretary must cut back the domestic programme to \$50 million a year. In the House of Representatives, George Brown (Democrat, California) is expected to introduce a parallel fusion bill next month; although the two differ on tactics, both Johnston and Brown are determined to extract a cast-iron commitment from the administration to ITER. "If we are going to proceed we need to establish some undertakings in the very near future,"

says Brown. "If the administration doesn't give a very strong indication of support, the project is going to die a natural death."

At the Princeton Plasma Physics Laboratory, positive results from a series of fusion experiments that have been running since December at the Tokamak Fusion Test Reactor (TFTR) have failed to allay concern about what will happen when the facility shuts this autumn. The reactor is due to be decontaminated and rebuilt as the TPX; but

the physics team at Princeton will face a long wait before the new facility starts to produce any data.

The TPX would need around \$120 million for construction in both 1996 and 1997 to be completed on schedule in 1999. Energy department officials admit they cannot see where this money will come from. So the project, if it does proceed, faces flat funding, increasing overall costs and delaying completion.

Yet the United States will not be able to choose between TPX and ITER. "They answer very different experimental questions," says Anne Davies, director of the Office of Fusion Energy at the energy department.

Some critics contend that TPX, ITER and other fusion experiments based on the Russian tokamak design are misdirected. "They've shut out competing approaches," says Arjun Makhijani of the Institute for Energy and Environmental Research in Takoma Park, Maryland. Makhijani suggests that more basic plasma physics research could provide less problematic solutions than those to be tested at ITER.

But Paul Rutherford, an associate research director at PPPL and chair of ITER's technical advisory panel, says that other concepts "have been tried and failed". The triumph of the tokamak, he says, has been "a matter of survival of the fittest".

Back in 1975, fusion experiments could yield just one-tenth of a watt of power out from 200 kW in, says Dale Meade, deputy director of PPPL. Progress since then has culminated in the recent experiments at PPPL, producing one-third as much energy out as went in.

ITER is designed to be fifteen times better than that, reaching the 'ignition' point at which 20 per cent of energy output is carried by alpha particles, sufficient to sustain the fusion reaction. The remaining 80 per cent of energy output is carried out of the reaction by neutrons, and could be used to generate heat and electricity.

Colin Macilwain

Networks and space gain in Japanese budget

Tokyo. Despite one of the most austere budgets on record, Japan's science-related ministries have managed to win significant new funding in a number of areas — including computer networks, space technology and university research grants — in the 1994 budget adopted by the cabinet last week.

The overall context is tight. Falling tax revenues, resulting from the prolonged recession, have forced the Ministry of Finance to trim back total government expenditure to ¥73,000 billion (US\$690 billion), only 1 per cent higher than last year.

The Ministry of International Trade and

Industry (MITI) has been particularly badly hit. Its budget request for science and technology was cut by ¥20 billion to ¥281.9 billion, only 0.4 per cent up on 1993. Reduced energy consumption because of the recession has led to a drop in revenue from taxes on electricity, and this in turn has forced MITI to accept a reduction in the funding it receives from this source.

The Science and Technology Agency (STA) fared better, with a 4.1 per cent increase. This is partly because the agency participates in large international programmes, such as the US space station and the International Thermonuclear Experimental Reactor (ITER), that cannot easily be cut back.

STA officials are pleased to have won their full request for ¥1.1 billion for a new interministry computer network linking national research institutes and universities, as well as providing a high-capacity link to overseas. This demonstrates the importance the government now attaches to developing Japan's networks, which lag far behind those of the United States.

This money comes on top of a further sum of between ¥7 and ¥8 billion to be spent on new local area networks (LANs) at Japan's national universities, announced by the government two weeks ago as part of the third 1993 supplementary budget. As a result of this and earlier supplementary budgets, all of Japan's 98 national universities will have campus LANs in the near future, whereas only half a dozen major universities had them a year ago.

The STA's spending on space will continue to grow. Expenditure will include ¥4.8 billion for the development of the unmanned space shuttle HOPE, an 18-metre vehicle expected to be launched around the end of the decade with Japan's H-II rocket, which was successfully launched for the first time earlier this month.

The budget for Japan's contribution to the US space station will grow to ¥46.8 billion, and a further ¥18.3 billion will be spent on the Advanced Earth Observing Satellite (ADEOS), which will carry sensors from the United States and Europe.

The Ministry of Education, Science and Culture (MESC) continues to inject large amounts of extra money into competitive grants for university research. With ¥82.4 billion in 1994 — 12 per cent up on last year — the ministry remains well on target for achieving the goal set in 1992 of nearly doubling the budget to ¥100 billion in a few years. But this budget still remains

small compared with other advanced nations.

MESC has also succeeded in getting an appropriation of ¥2 billion for a B-meson factory to be built at the National Laboratory of High Energy Physics (KEK) in Tsukuba. KEK researchers hope this will eventually become an international project involving high-energy physicists from around the world.

Japan's spending on the International Human Frontier Science Program, which supports international research on the brain and biological functions through a foundation in Strasbourg, France, has been cut by nearly 9 per cent to ¥3.6 billion — the first cut since the foundation was set up in 1989.

MITI officials point out, however, that although the budget has decreased when measured in yen, if the present high exchange value of the yen is maintained, the 1994 budget will rise by nearly 9 per cent over 1993 in dollars to \$34 million.

The prospects for future expansion of the programme's budget will depend on increased contributions from other participant nations. Last year, European countries, Canada and the United States contributed about \$8 million, and MITI officials are confident that, with the help of the new director-general of the programme, Michel Cuenod, the European Union will at least increase its contribution in 1994.

David Swinbanks

Is Japan throwing good money after bad science?

Tokyo. Despite the general squeeze on budgets (see above), the Japanese government apparently feels that it can afford to boost the budgets of two programmes of questionable scientific value, one into earthquake prediction and the other into cold fusion.

The first of these has come under severe criticism in recent years for its inability to predict earthquakes. External reviewers have raised fundamental objections to the programme, including questioning whether earthquake prediction is scientifically possible (see *Nature* 364, 370; 1993).

But the programme, now entering its seventh five-year phase, seems destined to carry on much as before, and with even more money. This year it will receive a 30 per cent boost to ¥10.6 billion (US\$100 million) for the six participating ministries and agencies.

Kiyoo Mogi, head of the coordinating committee for earthquake prediction, described earthquake prediction as a "100-year programme" and said "we should not give up even if we don't have immediate results".

Another anomaly is the Ministry of International Trade and Industry's (MITI's) plan to spend ¥540 million (\$5.1 million) on cold fusion research, more than twice the budget for 1993. The programme, euphemistically called research into "new hydrogen energy", will be carried out at a new laboratory recently established in Japan's northern island of Hokkaido.

The laboratory has 6 or 7 researchers drawn from Japanese companies, including Hitachi, Toshiba and Mitsubishi Heavy Industries, and MITI hopes to increase this number to 9 or 10 in fiscal year 1994, which starts on 1 April. The laboratory, which has been operating for only a few months, has yet to produce any evidence of cold fusion.

Japan's increased investment in earthquake prediction and cold fusion illustrates the lack of any effective system for reviewing government research projects. **D. S.**

Highlights of Japan's research budget for 1994

	Billion ¥	% change from 1994
MITI		
Industrial technology for global environment (RITE)	12.0	+19.3
Real World Computing (RWC)	5.0	+38.3
5th generation computer	1.4	+1.4
Intelligent Manufacturing System (IMS) Project	1.3	+12.5
Regional technology development	1.6	+135.9
DNA analysis	0.4	+38.1
New hydrogen energy (cold fusion)	0.5	+145.5
Total R&D budget	281.9	+0.4
STA		
Space	156.2	+7.9
Nuclear energy/safety	234.0	+1.7
SPRING-8	10.6	+17.8
Special promotion funds	15.5	+16.5
Human Frontier Science Programme	3.6	-8.9
Interministry computer network	1.1	-
Total R&D budget	605.2	+4.1
<i>(Includes 1.5 billion ¥ from MITI)</i>		
MESC		
Grants-in-aid of research	82.4	+12.0
Computer networks	29.6	+10.9
Accelerator physics (TRISTAN and B Factory)	11.8	-9.2
Astronomy (Hawaii telescope)	4.8	+20.0

Australia set to double medical research spending

Sydney. Government plans to double funding for biomedical research in Australia may be accelerated, following the publication of a report that strongly criticizes the funding and administration of the National Health & Medical Research Council (NHMRC), the main source of funds for such work.

The government had already promised in the last election campaign to double its spending on medical research by the end of the decade. Hints that the date for achieving this goal may be brought forward have now been made by Graham Richardson, the Minister for Health.

Commenting earlier this month on the NHMRC review, Richardson said it was "hazardous" to make promises in advance of discussions about the government's annual budget. But he added that the next annual budget, to be announced in September, would go very close to achieving that promise "within the next four to five years".

The review of the council, which advises the government on the allocation of \$A112 million (US\$80 million) in grants each year, was carried out by John Bienenstock, dean of health sciences at McMaster University in Ontario, Canada.

Bienenstock points out in his report that Australia spends less on medical research, both as a proportion of gross domestic product and as a proportion of the amount spent on health care, than any other country belonging to the Organization for Economic Cooperation and Development (OECD).

This low level of support is compounded by the lack of private sector support. Bienenstock also strongly criticizes the small size of NHMRC's secretariat in comparison to the value of the grants it administers. A lack of administrative support is the major factor in the council's failure to develop a strategic plan for health research, he says, or to address national health problems in particular areas, notably Aboriginal health.

His report concludes that the secretariat's support had lagged so far behind the growth in the council's activities that it "seriously jeopardizes the reputation and effectiveness of the NHMRC".

Ian McCloskey, chairman of the council's medical research committee, says that Bienenstock's report — including its comments on the size of the council's secretariat — was welcome. He said the amount spent on the secretariat was equivalent to 1.7 per cent of the total amount of grants allocated, and that the equivalent bodies responsible for medical research funds in other countries usually received between four and five per cent of the total value of research grants.

Mark Lawson

EMBL spreads future planning in bid to keep Italy on board

Munich. A special meeting of the European Molecular Biology Laboratory (EMBL) council last week took two steps that it hopes will help to persuade Italy to reverse its decision to resign from the laboratory at the end of this year.

The council agreed that Fotis Kafatos, EMBL's new director, should involve scientists from its member states more closely in the design of future science programmes. It also endorsed the laboratory's enhanced efforts to hire more staff from southern Europe.

Italy's resignation was announced at the end of December, and followed complaints over several years that it has relatively few staff positions at EMBL's laboratory in Heidelberg, despite paying 16 per cent (around DM10 million) of the laboratory's annual budget (see *Nature* 366, 604; 1993).

Kafatos had hoped to head off Italy's move by announcing a new programme under which research groups will be set up by EMBL in various countries in Southern Europe. Everyone, including the Italians, seemed satisfied, and the EMBL council meeting last December approved the setting up of four such groups in Italy, and one in Spain, for a five-year period from 1995.

But within weeks, Italy carried out its threat to resign, claiming that commitment to the regional groups was not as extensive as hoped. The resignation letter, however, made clear that Italy would be willing to rejoin if it received a better return on its contributions to the laboratory.

Now, in a move designed to broaden consultation over the development of EMBL's scientific programmes and also meet criticism of past procedures, the next five-year plan — to run from 1996 to 2000 — will be circulated in draft form to member states for discussion with their national scientific communities. Comments and counter-proposals will be considered by Kafatos before the plan is finalized, it is hoped by the middle of next year.

In addition, under a recruitment scheme introduced nearly a year ago and formally endorsed by council last week, a laboratory search committee will actively seek applications for its scientific posts from all parts of Europe, including those that seem to have been neglected in the past.

The council published a declaration stating that there will be "no question of compromising quality or introducing quotas". But, according to Steve Fuller, a senior scientist, the relatively high turnover of staff at EMBL, averaging four to five group leaders each year, combined with the efforts of the search committee, means that the balance of staff scientists "could

be changed quite quickly".

Italy is also taking steps to find out how much it is to blame for its underrepresentation at EMBL. Many Italian scientists complain that one reason is that young researchers are discouraged from spending long periods gaining experience abroad, as they are in danger of losing important contacts (see *Nature* 367, 590; 1994). An internal inquiry has been set up by the research minister, Umberto Colombo, to look into the situation, assisted by Italian members of the



Cold prospects: EMBL at Heidelberg.

European Molecular Biology Organization (EMBO).

EMBL is also taking a critical look at itself, and is preparing an analysis of the past 20 years to identify its successes and failures, in order to develop a more effective long-term role.

Uncertainty hangs over EMBL's future plans until Italy decides whether to withdraw its resignation. Last week, the EMBL council approved a 2.1 per cent increase in this year's budget to DM67.4 million, of which Italy will pay DM10.7 million. But the laboratory cannot begin to draft a budget for 1995, the last year of its current five-year plan, unless it knows whether Italy is definitely in or out.

At present, EMBL is proceeding on the assumption that Italy will stay in the fold. Preparations for all five of the new regional groups are going ahead, and the planned consultations on the next five-year plan will include Italy.

In case Italy does leave, however, Kafatos has already asked senior scientists at the laboratory to make savings in their 1994 budget totalling about a third of Italy's contribution, primarily by delaying the replacement of equipment and certain categories of staff, to help pay the deficit in 1995.

The 14 remaining member states will be asked to provide the rest to make up the missing budget. Salary rises for this year have been kept down to 1.6 per cent, rather than the expected 3.5 per cent. "But no-one is complaining about wage rises in the current climate," says Fuller. **Alison Abbott**

Nobel laureate rejects drug company charges

Munich. Allegations that the Italian pharmaceutical company Fidia "bought" the 1986 Nobel prize for medicine for the Italian neuroscientist Rita Levi-Montalcini have provoked a rush of emotional denials from Italy's scientific community.

Levi-Montalcini was awarded the prize jointly with the US scientist Stanley Cohen for their work on nerve growth factors, carried out in the United States in the 1950s. She is widely revered in Italy as a symbol of scientific achievement and integrity.

The allegations have been made by Duilio Poggiolini, former head of the health ministry's pharmaceutical section, who was arrested last autumn on charges of soliciting and accepting bribes from pharmaceutical companies in exchange for the registration and 'suitable' pricing of their drugs (see *Nature* 364, 663; 1994).

The ring of corruption is claimed to have included health minister Francesco De Lorenzo, many leading industrialists from pharmaceutical companies, and academics who acted in 'advisory' capacities.

Poggiolini claims that Francesco della Valle, director of Fidia until 1991, told him that he had paid out 14 billion lire (US\$8.4 million) to assure the prize for Levi-Montalcini. He says that part of the money was used to support her research, part to encourage other scientists to support her nomination, and part was given in bribes to the Nobel committee. Della Valle has denied the charge.

Nils Ringertz, general secretary of the

Karolinska Institute in Stockholm, whose Nobel Assembly awards the prize for medicine, says that its Nobel committee is not taking the allegations seriously. The procedure for selecting prizewinners is so long and complex, he says, that it would be impossible for pressure from any one source to be successful.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Levi-Montalcini: no basis to charges.

Levi-Montalcini herself has rejected the allegations against Fidia, claiming that the process for awarding Nobel prizes is "so complex that it cannot be corrupted".

Despite such assurances, Italy is taking the affair very seriously. It is regarded as a blow to the country's scientific credibility, and one which could damage further a drug industry already on the verge of collapse in the wake of the drugs scandal. Levi-Montalcini has received messages of support from all directions, including Italian president Oscar Luigi Scalfaro, Umberto Colombo, the minister of research, and many individual scientists. Colombo described the allegations as "slandorous", claiming that they are "denigrating to Italy's scientific community" and "seriously damaging to pharmaceutical concerns that are strongly committed to research and innovation".

The possible motivation behind Poggiolini's claims is difficult to guess. Levi-Montalcini is quoted in the Italian press as suggesting that multinational drug companies want to destroy the credibility of the entire Italian pharmaceutical industry to secure their own position in the Italian market. She claims that this theory has wide support, and also that the allegations are being used to discredit Italian science.

In a public statement, Colombo also hints at possible conspiracy. "One may wonder why accusations of this kind are being put about at just this critical juncture, when scientific research might provide the thrust needed to revitalize the nation's economy," he says darkly.

The allegations form part of a package of claims from Poggiolini, who is said to have maintained silence between his arrest last September and the end of January. He then implicated five executives from Italy's pharmaceutical companies in the drugs scandal, claiming that they had paid him bribes to assure the place — and price — of their drugs on the Italian market. Warrants against the five were issued last week, and four are already under arrest.

The four judges in Naples who are investigating the whole drugs scandal say in a joint statement that allegations that Fidia "bought" the Nobel prize are not central to their case. But they have not ruled out the possibility that they may seek permission to carry out investigations in Sweden at a future date.

Alison Abbott

White House urged to build support for global projects

San Francisco. The United States should ask the group of seven top economies (G7) to set up a panel to consider plans for major new international science facilities, and to recommend how these should be distributed between countries, according to Representative George Brown (Democrat, California).

The proposal is part of a three-stage plan to rescue the tarnished reputation of the United States as a reliable partner in international scientific collaborations, which Brown, who chairs the science, space and technology committee in the US House of Representatives, presented last Sunday (20 February), to the annual meeting of the American Association for the Advancement of Science (AAAS).

But the plan has a long way to go. "US government policies towards international collaboration are disastrous and incoherent," says Rodney Nichols of the New York Academy of Sciences. Tight budgets make that difficult to improve, he adds.

Brown said that the first step he is pro-

posing is that Congress should prepare legislation to confirm its approval in principle of all proposed research projects worth more than \$50 million. This would not guarantee funding for such projects; but it would require Congress to commit itself clearly to a project in advance.

He also wants the president's science adviser to prepare a report summarizing all the big science projects requiring international collaboration up to the year 2010. The president would then take this information to the G7.

Brown's staff have been investigating options for international collaboration in the wake of the collapse of the Superconducting Super Collider (SSC) and mounting concerns about the space station. He concedes that the last two proposals are vague, but says it would be up to the administration to provide the details.

"The only part we in Congress have control of is authorizing the big projects," he says. "The administration ought to be taking the lead on the other parts, and then

asking Congress to assist with a framework of law to help them do what they need to do."

Brown and other key figures now say that several projects, such as the International Thermonuclear Experimental Reactor (see page 669), the Large Hadron Collider at CERN in Switzerland and a future linear collider for high-energy physics, must be considered jointly, with sites divided between the countries paying for them.

As the director of the Stanford Linear Accelerator Center, Burton Richter, told a AAAS symposium on the aftermath of the SSC: "We are not going to get them one at a time. If you decide on three projects at once, I think you'll have an easier task."

But Brown is not optimistic that the current administration will give the support needed to get any such projects off the ground. "Everything is in turmoil right now," he says. "If our national research and development effort continues to go downhill, we are not going to be in very good shape to talk about international collaboration on big science."

Colin Macilwain

Cost concerns blamed for AIDS test hold-ups

Paris. François Gros, one of France's leading biologists and a former director of the Institut Pasteur, last week hit back at charges that in 1985 he and other officials delayed approval of an AIDS test marketed by Abbott Laboratories to protect the national market for a French test.

This so-called 'second' blood affair, which concerns an alleged delay in introducing routine screening of blood for antibodies to HIV, is distinct from an earlier case in which two officials were imprisoned for distributing unheated clotting factors contaminated with HIV to haemophiliacs, when they could have imported heat-inactivated products.

The new allegations were made in the newspaper *Libération* by Bernard Seytre — the translator of Robert Gallo's book *Virus Hunters* — and are based on memoranda written by Gros and other government advisers in 1985 which have been leaked from a continuing judicial investigation.

Seytre claimed that other documents also show that officials delayed the Abbott test both because the test marketed by the French company Diagnostics Pasteur was not working properly before June 1985 and because the company was not yet in a position to meet demand. Diagnostics Pasteur denies both allegations.

Official documents reveal that the National Health Laboratory (NHL), France's equivalent of the US Food and Drug Ad-

ministration, registered Abbott's application on 11 February 1985 and approved it on 24 July (the test had been approved in the United States on 2 March). The application from Diagnostics Pasteur was received on 28 February and approved on 21 June.

Memoranda also confirm that an interministerial meeting on 9 May 1985 chaired by Gros — who was then scientific adviser to Laurent Fabius, the prime minister — discussed at length whether to protect the Pasteur test.

An official from the health ministry told the meeting that a decision had to be taken quickly because the NHL "cannot hold back Abbott's application much longer beyond 13 May, the legal limit, without running the risk of a contentious appeal."

Gros admits that he tried to protect the Pasteur test, and says that there was concern that Abbott was making an aggressive attempt to invade the French market. "In the circumstances, it didn't appear to me to be shocking to keep a part of the French market for Pasteur," he says.

But Gros vigorously denies the main charge, namely that the government delayed introducing routine screening of blood because of this, arguing that the allegations cover only part of a more complex picture. He claims, for example, that the major consideration was not protectionism but cost, and that at the meeting on 9 May, the ministries of both finance and social affairs

refused to meet the estimated FF200 million (US\$32 million) annual cost of screening from health insurance funds.

Gros says this reluctance was partly due to an underestimation of the risk of post-transfusional AIDS; only four cases had

IMAGE
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François Gros: denies impropriety.

been recorded in France in 1985. He also says that Fabius and others were concerned about the consequences for people who tested positive.

This was a worldwide concern in 1985. Peter L. Page, of the American Red Cross Blood Service, wrote to *Nature* at the time suggesting that licensing should not occur until regional blood centres were able to implement the test with reasonable assurance that the number of false positives would be minimal, and the significance of anti-HTLV-III test positivity was clearly understood (see *Nature* 313, 824; 1985). Page also warned that the available tests "could cause undue concern for some donors, while not removing from the blood supply all units potentially infective for AIDS".

Gros adds that some officials were concerned that the level of false positives could require too many blood donations to be discarded, and thus disrupt blood stocks.

Fabius announced the introduction of systematic screening of blood on 19 June 1985, and most transfusion centres began on 1 August, making France one of the first countries to introduce screening. The UK government for example, introduced screening in the autumn of 1985 only after completing an evaluation of five AIDS tests in midsummer (see *Nature* 316, 474; 1985), the outcome of which favoured a British test marketed by Wellcome over the Abbott test.

What role — if any — protectionism by the French government played in the timing of the introduction of routine screening in France will probably be hammered out in court, as charges have been brought against Gros and other advisers relating to the affair. One of the questions to be answered is why the prime minister's office intervened in the procedures of the independent NHL.

Declan Butler

Delays 'fatally undermine' French claims

Paris. The lawyer who acts for Robert Gallo claimed last week that allegations that the French government delayed the Abbott AIDS test to allow a French test to be released first (see above) "fatally undermine" France's attempts to renegotiate its 1987 settlement with the United States which provides for an equal split of royalties from the patent on the AIDS blood test.

This arrangement was initially reached following agreement that Gallo, of the US National Institutes of Health, and Luc Montagnier of the Institut Pasteur be acknowledged as 'co-discoverers' of the AIDS virus. Although a settlement cannot legally be renegotiated, French officials have argued that the United States is "morally" bound to give it a bigger share of the royalties, on the grounds that Gallo's published sequence is now acknowledged to be virtually identical to the French strain, which was itself contaminated.

"Why should the US think it owes the French anything when they barred the US blood test from the French markets for narrow commercial reasons, killing their own citizens?" asks Joseph Onek, of the Washington law firm Crowell and Moring.

Onek says he intends to bring the newspaper articles describing the actions of French officials to the attention of the US administration, and "point out the extraordinary audacity of the Pasteur".

But the delay to the Abbott test has not been proved to have held up the introduction of routine screening in France (see above). Moreover, while Seytre claims that the French test was unreliable until June 1985, Françoise Brun-Vézinet of the Claude-Bernard hospital in Paris — whose data are cited by Seytre — says that, although there was some variability between batches, the quality of the test itself was not a problem.

Nonetheless, the Diagnostics Pasteur test does not appear to have been ready when the company submitted it for approval on 28 February. "We were still testing it in April," says Brun-Vézinet.

Whether the new charges will affect negotiations between the French and United States on the AIDS patent is another matter. Despite French sabre-rattling, Montagnier has said that the chapter is closed, and France's conservative government is said to have little interest in pursuing the matter.

D. B.

Britain unveils four-year plans for postgraduate studies

London. Virtually all students aiming for a PhD will have to spend four rather than three years in postgraduate study to qualify for a research council grant, according to detailed proposals published by the British government last week. But the first year need not be a formal course, providing the material that would have been taught on such a course is covered in other ways.

The plans, prepared by the Office of Science and Technology (OST), put flesh on a proposal first made by the Advisory Board for the Research Councils and endorsed in last year's white paper (policy document) on science. Their aim is simultaneously to broaden the scope of postgraduate studies, to ensure their content is closer to the needs of potential employers and to provide a suitable exit point after a year for those who feel they are not cut out for research.

As in the white paper, the government aims to achieve these objectives by introducing a new master of research (MRes) qualification considered suitable both as an introduction to a full research training and — for those who choose not to take this route — as a valuable qualification in its own right for those entering other scientific careers.

But whereas the white paper suggested this qualification be awarded only to those who complete an initial one-year course, containing an introduction to issues such as the communication of science, as well as a basic training in research techniques, proposals are more flexible, allowing the MRes to be awarded to those completing the first year of a four-year PhD programme.

The new option is being seen by some as a response to pressure from some of the 'old' universities. These had claimed that they lack the resources to introduce a wide range of new postgraduate courses, and feared losing PhD students to other institutions if such courses became compulsory.

The OST proposals, which have been sent to a wide range of institutions for comment, also respond to pressure from university departments in subjects such as physics and engineering which are currently developing four-year undergraduate courses. They suggest that students completing such courses might be exempt from the MRes requirement.

They also suggest that students who have acquired equivalent training in other ways — for example through a period of employment in industry — might also be exempt from the full four-year PhD.

OST officials argue that the new proposals are intended to move Britain away from the idea of seeing the PhD primarily as a

research 'apprenticeship' and bring it more into line with other countries — in particular the United States — which require completion of a master's course before embarking on postgraduate research.

The additional flexibility has, in general, been welcomed by universities and other bodies that have acknowledged the benefits which the MRes scheme could bring, but had initially been concerned that excessively rigorous requirements (for example on the content on taught courses) could become counterproductive.

The new proposals are "certainly a move in the right direction", says Joe Vinen, professor of physics at the University of Birmingham and chairman of a committee set up by the Royal Society to report on the future of postgraduate education. His remarks are echoed by the Committee of Vice Chancellors and Principals. "The general feeling is hopeful, even though we still have some concerns about the flexibility of the MRes," says its spokesman, Ted Nields.

But several worries remain. A spokesman for the Association of British Pharmaceutical Industries — which had previously opposed the four-year PhD suggestion, arguing that the current arrangements are adequate to meet the industry's needs — says the organization is still unable to see the relevance of the MRes degree to the pharmaceutical industry.

Others are concerned at the career prospects for those holding an MRes. "It could be seen as a failed PhD," says Colin Spedding, president of the Institute of Biology.

There is also a widespread worry that, in the absence of any additional funding from the government, the shift from three- to four-year postgraduate studies is likely to lead to a significant reduction in the number of PhDs produced by British universities, as well as a cut in the overall amount of research being carried out.

OST officials appear relatively unconcerned at this prospect. They argue on the one hand that a PhD may not always represent "value for money", either for the student concerned or for the government paying his or her costs; and, on the other, that it is "up to the research councils" to decide how to allocate their funding between support for research and postgraduate training.

But Paul Cottrell, assistant general secretary of the Association of University Teachers, says he does not accept that there is an argument for reducing the amount of public support given to PhD students. "This arbitrary decision to reduce numbers doesn't seem to have any basis, either market-led or otherwise," says Cottrell.

David Dickson and Fiona Gammie

Dramatic growth forecast for UK biotechnology firms

London. Britain's biotechnology sector is poised for dramatic growth, with a planned 44 per cent increase in staff and a 56 per cent rise in research and development (R&D) spending over the next three years, according to a report released in London this week by accountants Arthur Andersen and the UK BioIndustry Association.

At the end of 1993, Britain had 166 start-up companies in biotechnology, with a total turnover of some £438 million (US\$700 million) and R&D spending of £132 million. By 1996, Arthur Andersen forecasts that turnover by existing companies will increase to £878 million, while R&D spending will be £206 million.

Companies developing biotechnology-derived therapeutic agents will become the dominant force in the UK sector in this period. British biopharmaceutical companies at present have a total turnover of £194 million, and this is likely to rise to £319 million in 1996. At that time, they expect to be spending £147 million on R&D and employing more than 2,750 people.

The same trends are true across the board. As revenue among existing companies grows, so will the number of employees. The total workforce of the UK biotechnology sector at the end of 1993 was 7,400, and this is forecast to increase to 10,700 by 1996.

These numbers are conservative, as they do not include any projections for companies likely to be formed between the autumn of 1993 and 1996. Arthur Andersen predicts an explosion of new start-ups over the next three years.

Recent changes to listing requirements of the London Stock Exchange for biopharmaceutical and diagnostic companies is expected to persuade venture capitalists that investment in biotechnology is now an attractive option, as they can receive a faster return on their investments when the companies sell shares on the public market.

Achieving the kind of growth forecast by Arthur Andersen will require assistance. British biotechnology companies will have to increase their links with the United States and, further down the line, with companies based in other European countries and in Japan.

"All segments of the UK biotechnology sector regard strategic alliances as being crucial to the realization of their commercial objectives," says Vernon Spender, the author of the report. "Over the next three years the geographic spread of alliances will shift increasingly towards the US and Japanese markets as UK biotechnology companies seek to access those markets and the research and regulatory skills of their domestic companies."

Mike Ward

Report confirms obstacles to women scientists

London. Evidence of the British government's poor record in promoting women scientists to senior positions is expected to be revealed in a report on women in science to be published today (24 February) by the Office of Science and Technology (OST).

The report is expected to confirm concern raised in the government's white paper (policy document) *Realising our Potential* that women are "the country's biggest single most under-valued and under-used human resource". It shows that this is true not merely in scientific research, but also in the membership of public committees and advisory boards in fields related to science and engineering.

OST's two major committees, for example, the Council for Science and Technology (CST) and the Technology Foresight Steering Group, are made up of 21 men and just one woman, Bridget Ogilvie, director of the Wellcome Trust. The research councils emerge only slightly better. The four natural science research councils are made up of 76 men, and only six women (see table above).

The councils are being reorganized on 1 April, but so far no women have been named in key positions. Indeed, although these poor figures are in part inherited by William Waldegrave, the science minister, from his predecessors, there seems little evidence of any improvement over the past 12 months.

According to a parliamentary reply given

by Waldegrave earlier this month, for six of the top jobs filled by open competition during this period, there were 21 female applicants out of a total of 317, and none was successful. For ten research council posts, there were only two female applicants, and again neither was successful.

Last December, Helen Williams was appointed head of the trans-departmental science and technology group in the OST, a grade 3 post. She ended a run of 12 months up to the end of September 1993 when no females were appointed to any of the 24 senior OST posts at the grade 3, 4 or 5 levels which were filled internally.

"It is simply a case of going for the best person irrespective of gender," says Doreen Melville-Riddell a spokeswoman for the Office of Public Service and Science. She says that the Civil Service has a good record on equal opportunities, but that it is "not something you can change overnight by snapping your fingers".

But Michael Meacher, the Labour Party's spokesman on public services and science, who brought the government's recent record to light, says it is "incredible" that none of the women who applied for the vacant positions were considered appropriate for the job. "The government should be

taking a lead here," says Meacher. "I hope the OST report has some pointers, because William Waldegrave obviously needs them."

Bill Stewart, the chief scientific adviser to the government, has said in an interview published in the Association of University Teachers' *Bulletin* that the report looks at three broad areas; education — girls' inter-

Public appointments to science-related boards

	Men	Women	Dept
Council for Science and Technology	11	1	OST
Technology Foresight Steering Group	10	0	OST
Agricultural and Food Research Council	21	1	OPSS
Medical Research Council	18	2	OPSS
Natural Environment Research Council	19	1	OPSS
Science and Engineering Research Council	17	2	OPSS

OST, Office of Science and Technology
OPSS, Office of Public Services and Science

est in science and technology subjects; employment — reconciling a scientific career with family responsibilities and career breaks; and, women at the top — the number of women in senior positions and public appointments. The report is expected to be the most comprehensive study of the current situation for women pursuing scientific careers.

The report, which was originally expected last autumn, is thought to have been delayed by the reshuffling of the research councils. Nancy Lane, who is a member of the Women in Science and Technology Committee and also chairs the working group that compiled the report for the committee, says that "the white paper had to take prominence, and 'women in science' fell into second place".

The Royal Society has already set up its own separate study on the obstacles faced by women scientists. "The society's officers agree with the concern raised in the white paper, and have been examining the society's activities to identify ways of promoting and supporting women scientists," says John Horlock, the treasurer and vice president of the society.

One conclusion reached by the working group in a study of women scientists working in other countries was that networking is important. Although there are active groups in certain disciplines, such as the Women's Engineering Society, the Women in Physics Committee of the Institute of Physics and the Women Chemists' Committee of the Royal Society of Chemistry, there is no nationwide group of women in science. In particular, women in biology and biomedical science have no such group.

Joan Mason, the secretary of the working group, says that officials at the OST felt such an organization should be a 'grass-roots' movement. She has therefore arranged for the launch on 25 March in Cambridge of an umbrella organization, the Association for Women in Science and Engineering in the UK (AWISE).

Fiona Gammie

Coming to a screen near you...

London. In a move that marks the first step of a project to make the British Library's collection of historical manuscripts available to researchers across the world, images — such as that opposite — from an eleventh century manuscript of the poem *Beowulf* have been published electronically.

Lettering covered by paper mounts used to protect the document after it was damaged by fire in 1731 shows up because researchers used fibre-optic cable to backlight the document. Pages have been photographed in colour for the first time, and the ultraviolet light shows up erased corrections and microscopic detail in the parchment itself.

Selected folios from the manuscript are available on the Internet. The image files had to be compressed, as the originals are about 22 megabytes in size; as a result, they do not show the same high resolution. The entire manuscript will eventually be available in a variety of formats, including CD-ROM and network access, and visitors to the library will be able to call up the images on terminals.

"It is not yet possible to put everything

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out because the images are very large," says Andrew Prescott, curator of western manuscripts at the library. "But it does mark the first step." □

The British Library Board

Experiments and volunteers

SIR — I should like to respond to your News item "Nerve gas volunteers demand follow-up study" (*Nature* 367, 303; 1994).

The role of the Chemical and Biological Defence Establishment (CBDE) at Porton Down is to ensure that the UK Armed Forces have effective protective measures against the threat that chemical or biological weapons may be used against them. In order to carry out this work, it is necessary to use Service volunteers to assess the ability of Service personnel to function with new equipment and procedures; to develop medical counter-measures to protect Service personnel; and to evaluate the effects of very low and medically safe concentrations of chemical warfare agents on the ability of unprotected personnel to operate normally.

No studies involving volunteers are carried out unless there is a clear military need and a detailed protocol has been reviewed and approved by an independent ethics committee in accordance with the guidelines laid down by the Royal College of Physicians.

The procedure is that, on arrival at CBDE, Service volunteers are given a thorough medical examination to ensure that they are fit for the proposed study and are not suffering from any condition that might prejudice its results. The nature of the study is explained to them and they are told that they may withdraw without explanation at any stage and without detriment, penalty or pressure. The explanation about the study is given by those conducting it and by a military officer in a lay statement, in words that the volunteers will understand. They are advised how they may receive compensation in the unlikely event of their suffering any personal injury as a consequence of their having been volunteers at CBDE.

At the end of the study, the volunteers are given a second medical examination and the fact that they have participated at CBDE in a volunteer study is recorded on their medical records.

The practice has recently been instituted in which the lay statement read to all volunteers by a military officer states that "It is CBDE policy to call back some volunteer subjects for re-testing from time to time to ensure that techniques used give consistent and reproducible results and that no changes in the way we have applied the tests have occurred with time." From time to time, Service volunteers have been recalled so that checks on their medical health can be made. There is no particular frequency or pattern to such recalls. In addition, some volunteers return voluntarily to CBDE to take part in subsequent unrelated studies. The recall of any volunteers to CBDE is noted on their medical records.

When people leave the Services, their medical records are retained by the Ministry of Defence, but they are made available on request to the individual former serviceman or woman's doctor should the doctor wish to have access to them.

It is not in the national interest to publish details of volunteer studies as this information could be of significant benefit to a potential aggressor as he would be aware of the current state of our work to develop improved protective measures. However, during the past 30 years, the only nerve agent to which volunteers have been exposed at very low and medically safe concentrations was sarin, GB. Over the past 30 years, there has been no evidence that the health of Service volunteers has deteriorated as a result of their participation as volunteers at CBDE Porton Down. There are, therefore, no plans to recall volunteers other than those referred to above.

Graham S. Pearson

(Director General)

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Cyclotron future

SIR — The future of the South African National Accelerator Centre (NAC) at Faure is indeed under discussion, but we cannot agree with the tenor of Michael Cherry's article (*Nature* 365, 776; 1993) that the installation is doomed.

The most recent developments are much more positive. Keith Gottschalk and Roger Jardine, both members of the science and technology committee of the African National Council (ANC), are to visit NAC to form their own opinions. Although the outcome cannot be predicted, experts from Europe and the United States agree that NAC is outstanding in operational reliability and technological advancement. It is also one of the few particle installations operating a fully integrated hospital dedicated to cancer therapy.

NAC is one of the few facilities in the world at which the benefits of neutrons can be fully exploited, and more than 500 patients have so far been treated. More recently, South African clinicians and engineers have commissioned a proton-therapy facility and a unique beam-positioning system which is orders of magnitude faster in set-up time than any other system. The radiobiological support from the academic hospitals at Groote Schuur and Tygerberg has led to new insight about beam quality at depth and the necessary filtration. The physical

dosimetry group at NAC has made major contributions to beam simulation and the interrelationship between physical and biological effects of high linear energy transfer (LET) neutrons. Increasing use by experts from Belgium, Switzerland, Germany, Japan and the United States demonstrates that NAC already holds a respected position among particle therapy centres.

It is simplistic to suggest that closure of NAC would liberate R34 million for other scientific priorities. The real savings would be much smaller. Salaries at present approximate to R20 million and could not be abruptly stopped. A saving of R10 million would accrue from electricity consumption and general running costs, but this hardly justifies the closure of a successful institution.

NAC provides considerable benefits to South Africa far beyond that of academic training and research. Most of the cancer patients treated at NAC are underprivileged, and could be treated overseas only at enormous cost. Treatment at NAC is free. If NAC were to sacrifice isotope production and research altogether it could treat 200–250 patients a year. The present figure of 100 patients a year is in line with other dedicated centres with no commitment to other users.

Neutron therapy is an essential part of the radiotherapist's armamentarium, and 5–15 per cent of all patients needing radiotherapy will need neutron therapy. It has been predicted that the United States alone will need 25 proton-therapy installations.

It would be shortsighted if not outright foolish to abandon the first particle-therapy facility in the Southern Hemisphere.

Ben Smith

Lothar Böhme

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Oiling the waves

SIR — Daedalus has not carried the idea of using polymeric fullerenes as a lubricant for ships to its logical conclusion (*Nature* 367, 120; 1994). Why not synthesize a polymeric fullerene that will rotate in only one direction? This would convert the random motion of the water molecules to the ship in a unidirectional fashion, similar to the schemes for exploiting wave motion to generate electricity. Would this be a legal stratagem in boat racing?

Peter S. Tobias

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Religious debate continues

SIR — R. A. Savidge¹ and R. S. Courtney² criticize M. Vaneechoutte's carefully constructed hypothesis on the memetic basis of religion too strongly³. They have overlooked several points.

First, Christianity is only one of a large number of religions (including my own, Sikhism⁴). The fact that individuals in different parts of the world, left to themselves, have chosen to follow a variety of religions, suggests the strong desire to embrace such a meme. As a cynic once said, "God created Man, and Man, being a gentleman, returned the compliment".

Second, it is by no means clear that the Bible is a literal record of events that can be used as scientific evidence. Recent theological research suggests that much of the Bible is the result of wishful thinking by Jesus's followers⁵. Most of the miracles described (including turning water into wine) probably never occurred. A deep desire to believe in miracles, relics and places of pilgrimage appears to be a universal human trait; their widespread appeal appears secondary to meme-reinforcement.

Last, there is a large dichotomy between the preaching and practice of Christianity. The idea that only followers of the Christian faith have the opportunity to be "saved" (while the rest of us, presumably, go to Hell) appears to me to be a particularly self-centred example of meme-reinforcement. History does not abound with examples of Christians who "turned the other cheek". Instead, the physical elimination, or religious conversion, of those with conflicting memes seems to have been the norm.

To conclude, memes are essential to our security and psychological well-being; thus it is understandable that when a meme comes under threat the reaction varies from literary abuse to physical violence. This helps to explain why scientists who appear sensible in day-to-day life get so hot under the collar about their religion (or lack of it, for even atheism could be regarded as a meme. . .).

Vikramjit S. Kanwar

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SIR — One cannot but agree with R. S. Courtney² on peer review for pieces about religion. Thus the parable of the Good Samaritan⁶ is not necessarily about the compassionate care of a Samaritan for a Jew. In none of the four versions of the New Testament to which I have access (the Authorized Version, the New English Bible, the New Standard Version and the Good News Bible) is the man for whom the Samaritan cares identified as a Jew. Surely the point of the parable is that

a Samaritan (that is, a member of a lowly regarded Jewish sect) gives succour to the injured man whereas the priest and the Levite (Sadducees/Pharisees?) passed him by. So indeed the parable might be about compassionate care but not about non-Jew for Jew (possibly the reverse). Alternatively it might well be just scoring points against the religious establishment.

R. Leberman

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SIR — I would like to ask R. A. Savidge¹ whether he has a scientific proof that the transformation of H₂O into CH₃CH₂OH really happened. The New Testament was written in Greek by the four evangelists between the years 70 and 100 of the Christian era, about 40 to 70 years after the death of Jesus. It is embellished by hearsay, mainly of non-contemporaries, not of eye-witnesses. For a scientifically minded non-Christian and nonbeliever, the Gospel thus has no more credibility than any other ancient manuscript, Homer's *Iliad* for example. Moreover, it is not a documentary or historical record; its purpose, rather, was to propagate the Christian faith. Did it never occur to Savidge that some or even many events described in the Bible could be pure products of imagination, invented only to impress those to be converted?

Friedrich Katscher

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1. Savidge, R. A. *Nature* **366**, 606 (1993).
2. Courtney, R. S. *Nature* **366**, 606 (1993).
3. Vaneechoutte, M. *Nature* **365**, 290 (1993).
4. Cole, W. & Sambhi, P. S. *The Sikhs: Their Religious Beliefs and Practices* (Routledge and Kegan Paul, London, 1984).
5. Crossan, J. D. *Jesus: A Revolutionary Biography* (Harper, San Francisco, 1993).
6. Luke 10: 30–35.

SIR — I can go one better in cynicism than Ralph Estling (*Nature* **364**, 754; 1993). Maybe religion is just a method by which the weak and the poor try to persuade the strong to be gentle and the rich to be generous, with a sizeable cut going to the persuaders. To make it work, supernatural powers and survival after death must be postulated. Otherwise there is no reason for anyone to listen to such appeals: the powerful are beyond punishment here and now.

The scam apparently works, as all human tribes have some kind of religion. Great cultures have been accompanied by impressive religions. Atheistic societies have left no trace in history. The one recent attempt to form a religionless culture in the Soviet Union has failed.

Andrejs Baldins

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SIR — Having just read recent issues of *Nature*, I notice, with puzzled gloom, a letter advocating creationism. In the decades I have been a regular reader, I cannot recall that you ever printed a letter claiming that the Earth was flat, or that cattle disease was caused by malign sorcery; yet, ever and anon creationism has reared its silly head. Why, pray, is this particular variety of rubbish so uniquely privileged?

M. Hammerton

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SIR — Sir Hermann Bondi (*Nature* **365**, 484; 1993) asks how those who claim that "Christianity provided the necessary background for science" can account for "the fact that for three-quarters of the Christian era the home of science was confined to the non-Christian parts of the world". He ignores the work of many Christians in the early centuries that prepared the way for the achievements of the Renaissance. Thus, in the sixth century John Philoponus, a Christian Platonist who lived in Alexandria, wrote extensively on the material world. In his commentary on Aristotle's physics, his belief in creation led him to say, contrary to Aristotle, that projectiles move through the air because initially they are given a certain quantity of motion. He also rejected Aristotle's distinction between celestial and terrestrial matter. In these and other ways Christian beliefs led to the destruction of Aristotelian physics, thus opening the way to modern science.

Modern science is made possible by insistence on logical coherence and experimental verification. These were present in a qualitative way among the Greeks, and the vital contribution of the Christian Middle Ages was to refine these conditions into a more effective union by insisting on the quantitative precision that can be attained by using mathematics in the formulation of theories, and then verifying them not by observation alone but by precise measurements. This was achieved in the twelfth century, principally by Robert Grosseteste, one of the first chancellors of the University of Oxford, who is regarded as the founder of experimental science (see A. C. Crombie *Robert Grosseteste and the Origins of Experimental Science 1100–1700*, Clarendon Press, 1953). Subsequent work in Oxford by the Mertonian School, and in Paris by Buridan, Oresme and others, developed these early insights and spread them throughout Europe. This has been exhaustively documented by Duhem, Crombie, Maier, Jaki, Grant and many others. Thus it can be said that the foundations of modern science were laid in the Christian Middle Ages.

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Paternal exposure not to blame

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The "Gardner hypothesis" suggests that an excess of childhood leukaemia near a nuclear reprocessing plant is caused by paternal exposure to ionizing radiation. But the evidence shows that this explanation is wrong.

THE report 10 years ago of an unexpectedly large number of cases of leukaemia in young people in Seascale, a small town 3 km south of the Sellafield nuclear reprocessing plant in northwest England, has given rise to much public concern and much scientific research. The idea that the increased number of cases might have been due to local pollution from radioactive waste did not seem likely, and interest focused instead on an alternative explanation that has come to be called "Gardner's hypothesis". This hypothesis postulated that the men's exposure to ionizing radiation in the course of their work led to mutations in their sperm which increased substantially the risk of leukaemia in their children. The idea was appealing, and it led to a court case in which two families sought compensation from the company which operates the plant, British Nuclear Fuels. In the course of the case a great deal of new evidence became available which justifies the conclusion that the hypothesis is wrong.

Clusters near installations

The Sellafield plant started operations in 1950. Thirty-three years later it was discovered that seven cases of leukaemia had occurred in young people under 25 years of age who lived in Seascale in the period 1955–83. The expected number could not be calculated precisely, but the excess under 10 years of age was about tenfold (5 against 0.5 expected) and the cases have come to be known as the Seascale "cluster". No estimate of the probability with which such a cluster might have occurred by chance was possible, as the area in which the excess was observed had been defined *post hoc*. The finding was nevertheless disturbing, and many investigations were begun.

In one of these studies, Craft *et al.*¹ examined the spatial distribution of all cases of cancer in young people under 25 years of age in the north of England in the period 1968–85. Altogether they had data for 6,686 cases of cancer (1,272 electoral wards), and they examined the incidence using the populations recorded in the 1971 and 1981 censuses. Whichever set of population estimates they used, the excess of acute lymphatic leukaemia was the most extreme in Seascale (*P*, in each case, is less than 0.001). Another of the five most extreme findings occurred in Egremont, a small town 7 km north of Sellafield, and it is difficult to attribute to

chance the occurrence of another extreme excess in the same area (*P* with either population estimate is less than 0.01).

A third small cluster that is statistically less impressive has been described near Dounreay, the site of the only other nuclear reprocessing plant in the United Kingdom²; less impressive excesses have been reported around some other nuclear installations in the United Kingdom and in Germany.

Paternal irradiation

In their first study to seek an explanation for the Seascale excess, Gardner *et al.*^{3,4} examined the incidence of leukaemia and other types of cancer in young people born to mothers living in Seascale and another of young people who went to school in Seascale but had been born to mothers living elsewhere. Only fatal cases were included in the period 1950–70, because national incidence data before 1971 were too incomplete for a reliable estimate of non-fatal cases. For the subsequent period (1971–86), both fatal and non-fatal cases were included. Six cases of leukaemia were identified in the first cohort against 0.61 expected, and none in the second cohort against 0.57 expected.

These findings focused interest on young people born in Seascale, and Gardner *et al.* limited their subsequent case-control study to young people who had also been born there⁵. Their principal findings, suggesting that paternal irradiation before a child's conception might be responsible for the increased risk of the disease, are summarized in Table 1. Two sets of data are shown: those relating the

child's risk of leukaemia to total external dose received by the father before the child's conception; and those relating the risk to an estimate of dose received in the immediately preceding 6 months. Both sets show a significant excess with the highest doses; but only the latter shows a progressive increase with increasing dose. Gardner's hypothesis that these findings implied causality can be tested both by reference to what is known about the heredity of leukaemia in young people and the genetic effects of ionizing radiation and by seeing whether similar effects are produced elsewhere.

Mutational changes have been associated with some acute lymphoblastic leukaemias of children, and these are often identified by the presence of specific chromosomal translocations. The changes, however, are present only in leukaemic and not in normal cells. They are, therefore, acquired somatic mutations and not constitutional changes transmitted from parent to child via the germ cells. The idea that ionizing radiation could cause a gonadal mutation that caused a child to have leukaemia is, nevertheless, plausible, but has to be judged against the background of what is known about the heritability of childhood leukaemias and about the radiation exposures required to produce mutations in mammalian genomes.

Examples of possible familial patterns of childhood leukaemia are uncommon. According to the records of the National Childhood Cancer Register, 84 of the 9,000 or so children who developed leukaemia in the United Kingdom be-

TABLE 1 Relative risk of leukaemia in young persons by father's occupational exposure to ionizing radiation

Father's dose before child's conception	No. of cases		Relative risk	95% confidence limits
	Leukaemic children	Area controls		
Total				
0	38	253	1.00	—
1–49 mSv	3	19	1.12	0.31–4.05
50–99 mSv	1	11	0.69	0.08–5.73
≥100 mSv	4	5	6.24	1.51–25.76
Previous 6 months				
0	38	262	1.0	—
1–4 mSv	3	18	1.30	0.32–5.34
5–9 mSv	1	3	3.54	0.32–38.88
≥10 mSv	4	5	7.17	1.69–30.44
No dose	38	253	1.00	—
Any dose	8	35	1.71	0.68–4.26

From ref. 4

tween 1962 and 1992 had twin partners of the same sex, and in four pairs both twins were affected. If, as is usual, half the pairs of like-sex twins were monozygotic and all the four concordant pairs were also, this would imply genetic determination in about 10% of cases. Half the concordant pairs, however, were diagnosed in the first year of life and in such cases the disease has been found to have spread *in utero* from one twin to the other⁶. The true proportion of genetically determined leukaemia is therefore estimated to be about 5%. Other indications that the contribution of inherited mutations to childhood leukaemias must be small include the lack of evident increase in risk with paternal age and the rarity of childhood leukaemias in the offspring of survivors of the disease⁷.

This knowledge of the heritability of childhood leukaemia, and the fact that it occurs in about 1 in 2,000 children, allows the possibility that a recessive mutation with a low degree of penetrance contributes to the development of the disease and is present in a substantial proportion of the population. It effectively excludes, however, any major contribution from the type of mutation that would be required to account for the appearance of the Sellafield cases in the first generation: namely, a dominant mutation with a high degree of penetrance.

Knowledge of the mutation rates induced by radiation also weighs against the Gardner hypothesis. Information on such rates is available from *in vitro* studies on human and mammalian somatic cells in culture; from breeding experiments on irradiated animals; and from human populations exposed to radiation, in particular from the survivors of the atomic bombings at Hiroshima and Nagasaki. The mutation rate of a given gene depends on various factors, including its size. The highest induced mutation rate per cell observed in radiation studies on cultured somatic cells is about 1×10^{-5} per gene per Sv, or 2×10^{-6} for a dose of 200 mSv, the highest exposure level for the Seascale workers⁸. Studies on radiation-induced inherited recessive and dominant mutations in the mouse indicate that the acute dose required to double the spontaneous mutation frequency may be around 0.7–2.5 Sv, with the dose for chronic exposure being 2 to 3 times greater. The work of Neel and colleagues⁹ on children of the atom-bomb survivors, including more than 1,500 born to parents who received a gonadal exposure of 1 Sv or larger, revealed no clearly increased frequency of mutations and suggested a best estimate for the dose required to double the mutation rate in humans of around 2 Sv for an acute exposure and, by analogy with animal data, 4 Sv for chronic exposure. These doses are far in excess of those received by the Seascale workers.

TABLE 2 Risk of leukaemia in young people and father's irradiation before child's conception: studies with objective measurement of exposure

Reference	Any dose/unexposed		Relative risk	Cumulative dose 100 mSv or more/unexposed		Relative risk
	Cases	Controls		Cases	Controls	
5*	8/38	35/253	1.71 (0.68, 4.26)	4/38	15/253	6.24 (1.51, 25.76)
13†	5/17	10898/ 41079	1.11 (0.36, 3.19)	3/17	6717/41079	1.08 (0.25, 3.89)
14*	11/1104	27/3318	1.26 (0.59, 3.90)	0/1104	3/3317	0.00
15‡	6/106	53/837	0.87 (0.32, 2.34)	0/106	5/837	0.00
12§	3/51	2/322	9.0 (1.0, 107.8)	0/51	0/322	—

*Under 25 years; †under 20 years; ‡under 15 years.

§Leukaemia (47 cases) and non-Hodgkin's lymphoma (4 cases) under 5 years. The distinction between leukaemia and the much rarer non-Hodgkin's lymphoma in this young age group is sometimes arbitrary and both diseases have been grouped together in this study and some others^{8,18}.

As the incidence of childhood leukaemia in the United Kingdom is around 1 in 2,000, it appears from the study of monozygotic twins that the incidence of the disease due to penetrant dominant mutations is unlikely to be much more than 1 in 40,000, and it is a figure of this order that has to be compared with the incidence in Seascale if a germ-cell mutation(s) is proposed as a cause. A recent study has identified 107 children born to parents at Seascale who had received a radiation dose greater than or equal to 100 mSv of whom five developed leukaemia (L. Parker & R. Wakeford, personal communication). If these are presumed to have been a consequence of an inherited induced mutation, then the mutation rate of about 5% would have had to be about 2,000 times the background rate of about 1 in 40,000. Such a hypothesis would demand: (1) that a leukaemia gene(s) be uniquely and inordinately sensitive to radiation, as radiation damage is induced randomly in the genome and other well-known inherited single-gene syndromes, with high spontaneous mutation rates, are not increased in association with the increased leukaemias; and (2) that the gene(s) be selectively susceptible to occupational irradiation, as there is only a minor contribution of inherited dominant mutations to childhood leukaemia in the general population, which is exposed, on average, to about 30 mSv of natural radiation.

Other communities

Previous evidence bearing on Gardner's hypothesis had been limited to parental histories of the frequency of radiological examinations taken after the child's disease had been diagnosed: in such studies the results are liable to be affected by recall bias. In the Oxford Childhood Cancer Study (by far the largest study of the aetiology of childhood leukaemia), more of the fathers of the children with cancers reported that they had had a radiographic examination before the child's conception than the fathers of control children¹⁰. But

the fathers of the children with cancer reported relatively fewer radiographic examinations after the child's delivery, which compensated for the excess number before the child's conception, and the authors concluded that the reported timing of the exposure was biased by knowledge of the child's disease. In the only study which attempted to verify the parents' histories by checking hospital records and doctors' notes, the fathers of children with leukaemia were found to have had more abdominal X-rays before the child's conception than expected from the controls, but the excess was small and not statistically significant¹¹.

To have confidence in such a relationship it is necessary to have objective evidence of exposure. This has been obtained in four other studies (Table 2). Data are given only for total numbers of fathers exposed and unexposed and for the numbers having received a cumulative dose of 100 mSv or more before the child's conception. Gardner *et al.*⁵ found a somewhat closer relationship with the dose estimated to have been received in the 6 months before the child's conception than with the total cumulative dose, but doses in this period are of less interest for three reasons. First, the biological evidence indicates that the sperm and their immediate predecessors, which exist *in vivo* for up to 12 weeks, are only slightly more susceptible to mutational damage than the spermatogonia, so that the total cumulative doses would be expected to have a greater effect than the doses received in the 12 weeks before conception as they are much larger. Second, the 6-month dose recorded by Gardner *et al.*⁵ was an estimate obtained by halving the preceding annual dose; the actual data⁸ for the Sellafield employees show a less close (statistically non-significant) relationship between recent pre-conception doses and Gardners' leukaemias than is shown by the cumulative doses. Third, the only study to support Gardner's hypothesis provides it only when total cumulative doses are considered¹².

The clearest evidence is that provided by observations of the children of the survivors of the Hiroshima and Nagasaki atomic bomb explosions¹³. The lack of material excess in the children of the irradiated group is particularly striking, as the mean doses to the fathers' gonads was 406 mSv — substantially greater than the doses received by the children's fathers at Sellafield.

Two case-control studies are also negative. One¹⁴ included all cases of leukaemia that occurred in Scotland and a part of north Cumbria on the Scottish border in people under 25 years of age in 1958–90 born in Scotland in or after 1958, the year in which nuclear operations in Scotland began. The other study¹⁵ was modelled on that of Gardner *et al.*⁴ and covered all children under 15 years of age who were born near five nuclear installations in Ontario recorded in the Ontario Cancer Registry as having died of leukaemia in 1950–63 or of having developed it in the period 1964–88, when records of non-fatal cases were believed to be complete⁵. Neither set of results suggested the probability of a hazard from the father's occupation.

The third case-control study¹² was undertaken to explain a possible excess risk of childhood leukaemia near two nuclear sites in the south of England. The study was limited to children under 5 years of age, as the observed excess had been concentrated on cases at these ages. Paternal exposure, however, had little or no influence on the local leukaemia rates that had engendered the study as only 3 out of 47 children in the area with leukaemia for whom information could be obtained had fathers who had been monitored for radiation in one or other of the three plants, and the local rates implied an excess of about 18. It is still necessary, however, to ask whether some or all of these three cases should be attributed to paternal irradiation. The proportion of the fathers who were exposed was significantly greater than in the controls ($P=0.05$), but the doses were minute (less than 5 mSv). Even if the doses had been high, the excess of parents employed in the nuclear industry would have gone only a very little way towards explaining the local excess.

An alternative explanation^{8,12} is that external exposure served as an indication of a more important local dose to the testis from a radionuclide absorbed and deposited in the testis. In the Health and Safety Executive's study⁸ an association was observed between leukaemia plus non-Hodgkin's lymphoma (see Table 2) and potential exposure of the father to tritium before the child's conception, as judged by place and time of work (based on five exposed fathers). This was statistically highly significant and continued to be significant after controlling for residence at Seascale at birth ($P=0.027$). An asso-

ciation was also found with measured intakes of tritium in the most sensitive period 12 weeks before the child's conception ($P=0.005$) but not with the more important cumulative dose nor with measured intakes of alpha emitters or all radionuclides at any period (P values ranging from 0.24 to 0.74). No association was found with measured doses of tritium near the Canadian nuclear reactors¹⁵, where tritium provides the most common type of internal exposure.

Three other recent studies bear on the interpretation of Gardner's hypothesis. First, no excess of leukaemia in young people has been observed in the whole of the rest of Cumbria¹⁶ outside Seascale, despite the fact that 92% of the births to Sellafield employees occurred outside Seascale during the relevant period (1950–89) and the fathers of these children had received 93% of the total preconception dose¹⁷. The numbers are large, 779 births to Seascale residents and 8,482 elsewhere, with total collective doses of 38 and 501.4 man Sv, respectively. If the former dose had been responsible for the 4 Seascale cases associated with paternal irradiation, some 53 cases would have been expected in the rest of Cumbria where no excess was found, and 52 cases should have occurred in west Cumbria where Gardner *et al.*⁵ found 4.

Second, none of the fathers of the four children with acute lymphatic leukaemia (A. W. Craft, personal communication) who constituted the second cluster near in Egremont¹⁷, and only one of the fathers of the six children with leukaemia constituting the Dounreay cluster, had any occupational exposure to ionizing radiation.

Third, Kinlen¹⁸ has now found that the excess in Seascale is not limited to children born there, but has also extended to children born elsewhere but living in Seascale. This is contrary to the finding of Gardner *et al.* and is accounted for by the fact that, for various reasons, the three cases of leukaemia in Kinlen's series were not included by Gardner *et al.*. Only one of the fathers of the three children had received any occupational dose before the child's conception, and that was low (5 mSv).

The legal judgment

All the evidence discussed above, apart from that published by the Health and

Safety Executive⁸ and the personal communication from Parker and Wakeford, was available to the judge in the case that was brought against British Nuclear Fuels, although much of it was still in press at the time. (The Health and Safety Executive report was headlined in the press as providing new evidence in support of Gardner's hypothesis, although all it did, in this respect, was to confirm the accuracy of Gardner's report of a relationship with cumulative dose in the original cases.) On the evidence before him, the judge concluded that "the scales tilt decisively in favour of the Defendants and the Plaintiffs, therefore, have failed to satisfy me on the balance of probabilities that PPI [paternal preconceptional irradiation] was a material contributory cause of the Seascale excess"¹⁹.

Conclusion

In our opinion, the hypothesis that irradiation of the testes causes any detectable risk of leukaemia in subsequent offspring cannot be sustained. It does not accord with what is known of radiation genetics or of the heritability of childhood leukaemia. It is not supported by observation of the relationship between men's exposure to ionizing radiation and the risk of leukaemia in their offspring in the survivors of the atomic bomb explosions in Japan, in the neighbourhood of nuclear installations in Ontario, in Scotland, or in Cumbria other than Seascale. It makes no contribution to the cluster of young people in Egremont and cannot account for the cluster near Dounreay, nor for the excess near to nuclear sites in the south of England, nor even for the whole of the cluster observed in Seascale.

We conclude that the association between paternal irradiation and leukaemia is largely or wholly a chance finding. Nevertheless, it appears likely that small but real clusters of leukaemia in young people have occurred near Sellafield, and some other explanation for them needs to be sought. □

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Comfort for next century but one

Rumours of an impending calamitous collision of a comet with the Earth have been discounted by the use of ancient Chinese records to refine the orbit of the prograde comet Swift-Tuttle.

FEARS that the world will end on or about 14 August 2126 can now be put aside, at least for the time being. That is the immediate outcome of a recalculation of the orbit of Comet Swift-Tuttle, the comet whose debris is recognizable as the Perseid meteor shower, which was most recently observed in the weeks preceding its most recent perihelion passage in December 1992.

As comets go, Swift-Tuttle is a prime candidate for terrestrial collision because its perihelion distance is smaller than, but differs by less than five per cent from, the radius of the Earth's orbit. That means that, for some weeks on either side of perihelion, the comet moves in a trajectory never further than a few million kilometres from the orbit, roughly parallel with it and in the same direction as the Earth, but at greater speed. The chance of a collision obviously depends rather critically on the exact times at which the comet crosses the Earth's orbit. A few days either way can make a critical difference.

Whatever happens, those in a position to observe the night sky in the weeks preceding the next perihelion passage on 12 July 2126 are almost guaranteed the sight of a bright comet with an illuminated tail. The hope, for their sake, must be that the uncertainties in the recalculation due to Kevin Yau, Donald Yeomans and Paul Weissman of the Jet Propulsion Laboratory in Pasadena, California (*Mon. Not. R. astr. Soc.* **266**, 305-316; 1994) are not so great that there is a collision after all.

For those of us who will not be there to see the sights, the recalculation has the great interest of illustrating how to make bricks without straw — the exiled Israelites' daunting task. Until its apparition in 1992, Swift-Tuttle had been observed properly only in 1862, from Western Europe before perihelion and from the Cape of Good Hope in South Africa afterwards.

The best estimate of the orbit of the comet from those observations was made retrospectively in 1973 by B. G. Marsden. His calculation gave equal weight to the observations in the Northern and Southern Hemispheres, and raised the possibility of a collision with the Earth in 2126. Yau, Yeomans and Weissman, by contrast, are able convincingly to discard the Cape observations as likely to have been systematically in error by dredging more informative data from ancient records, notably from the Chinese.

By good luck, as it now appears, the

Jesuit missionary Ignatius Kogler, who ran Chinese astronomy for a quarter of a century until his death in 1746, recorded in 1737 the appearance of a guest-star in the sky between 7 and 16 July. That now seems to have been the last apparition but two of Swift-Tuttle. But even the Chinese records are innocent of earlier sightings of the comet for a millennium and a half, when a surviving record from the Han Dynasty describes a guest-star that appeared on 28 July 188, moved southwest over the sky on successive nights and then disappeared. And there is an even earlier record of a guest-star behaving in much the same way that appeared between 20 and 27 August in 69 BC.

The value of these early records for the determination of the orbit of Swift-Tuttle is comparable with that of a distant landmark when taking a compass-bearing: the more distant in time the putative apparition, the more accurately the free parameters in the orbital equation can be determined. But the decision to accept the Chinese records as authentic observations of the comet, and to discard the authentic observations of 1862 from the Cape of Good Hope on the grounds that they must have contained a systematic error, raises an interesting question in the psychology of the analysis of data.

The Chinese records may refer to quite unrelated phenomena, but Yau and his colleagues are impelled to regard them as genuine observations of the comet by the compulsion of coincidence. When they project their best trial orbit for the comet backwards two thousand years, they postdict apparitions in 69 BC and 188, just as the Chinese said. But there is other circumstantial evidence to make the argument convincing.

What, for example, happened to Swift-Tuttle between 188 and 1737? With a recurrence period of 130 years or so, it should have reappeared a dozen times in that long interval. Why was it not seen and recorded somehow? The explanation, based on the best orbit of Yau and his colleagues, seems to be that on each occasion it would have been too faint to be seen with the naked eye (and, of course, there were no telescopes before the seventeenth century).

The argument is that the comet is visible from the Earth only when within a distance of 0.6 astronomical units (one astronomical unit is the radius of the Earth's solar orbit). That means that it should have been just at the limits of visibility during perihelion passages in the years 59, 698 and 1079. Will other historians now take up the challenge

to find other records of guest-stars appearing in the sky in the northern summer months of these years? For their part, the authors wring their hands with regret that evidence of a visible apparition of 447 BC is likely to have been destroyed by the sacking of Hsien-Yang in 216 BC, if not by an earlier act of imperial vandalism.

Meanwhile, it is important that the orbit of Swift-Tuttle now provided is not a mathematically defined ellipse with large eccentricity, but a series of ellipses obtained by the numerical integration of the motion of the comet that allows for its gravitational interaction not just with the Sun but also with all the planets outside the Earth's orbit except Pluto (which is too small to matter).

In this simulation of the comet's motion, the gravitational forces are updated every notional three months. Inevitably, that technique reproduces many of the features of a chaotic system; the interval between successive approaches to perihelion jumps about, ranging from 127 years to more than 135 for example, while the authors acknowledge that there is little purpose in calculating the ephemeris of the comet beyond the next few orbits (or centuries).

No doubt that is also a prudent acknowledgement that, by the next apparition of Swift-Tuttle, there will be a still better description of the movement of the planets even than those now available, most spectacularly (on magnetic tape) at the Jet Propulsion Laboratory. Even so, the calculation of the past motion of Swift-Tuttle rests on a planetary ephemeris of 1970s vintage corrected to match later versions. Without a shadow of hesitation in their prose, the authors flatly say that there will not be a collision with the Earth at the next two apparitions, on 12 July 2126 and 11 August 2261.

That may be just as well, for the success with which the orbit of Swift-Tuttle accords with the ancient Chinese observations suggests that the comet is dynamically much less disturbed by the emission of gases under the influence of the Sun than is, for example, Halley's Comet (where the dynamical effect of outgassing adds several days to the period of the comet at each return). Swift-Tuttle is intrinsically as bright as Halley, suggesting that outgassing on the same scale has much less dynamical effect, most simply explained if the comet is much more massive than Halley.

John Maddox

How fast can you get?

Wolfhard Almers

NEUROTRANSMITTER release from a pre-synaptic terminal is one of the fastest things animal cells do — it occurs in a brief burst within the first millisecond of the arrival of an action potential^{1,2}. On page 735 of this issue³, von Gersdorff and Matthews explore calcium-triggered exocytosis of synaptic vesicles by capacitance measurements. They provide the most direct evidence yet that the calcium sensor for exocytosis is a low-affinity receptor, and demonstrate a hitherto unrecognized, and surprisingly fast, mechanism for endocytosis. They show that, for 100 ms or more, terminals can continue to release transmitter at rates close to those attained after an action potential (0.2 vesicles per millisecond per release zone).

The existence of a low-affinity Ca sensor had been widely postulated⁴. At the release (or 'active') zones of synaptic terminals, vesicles and voltage-gated Ca channels co-inhabit microdomains where cytosolic $[Ca^{2+}]$ rises in microseconds to near-millimolar levels as individual Ca

channels open, and where $[Ca^{2+}]$ falls nearly as quickly when the channels close. For release to stop rapidly after an action potential, the Ca sensor must let go of its bound Ca quickly. So its low affinity is of critical importance.

The rapid endocytosis revealed by von Gersdorff and Matthews coexists with the more familiar, slower, membrane retrieval probably mediated by clathrin-coated pits^{5,6}. Similar effects have also been seen after exocytosis of dense core granules⁷. In both cases, the slow mechanism is evident only after massive stimulation, whereas the fast mechanism probably operates throughout. Therefore, for the first time, we may have observed the physiological mechanism of membrane retrieval after Ca-triggered exocytosis in excitable cells.

The new results on the kinetics of release are perhaps the most intriguing, as they have implications for the number of vesicles that a synapse can release rapidly, as well as for the speed of the exocytic machinery. The issues involved are illus-

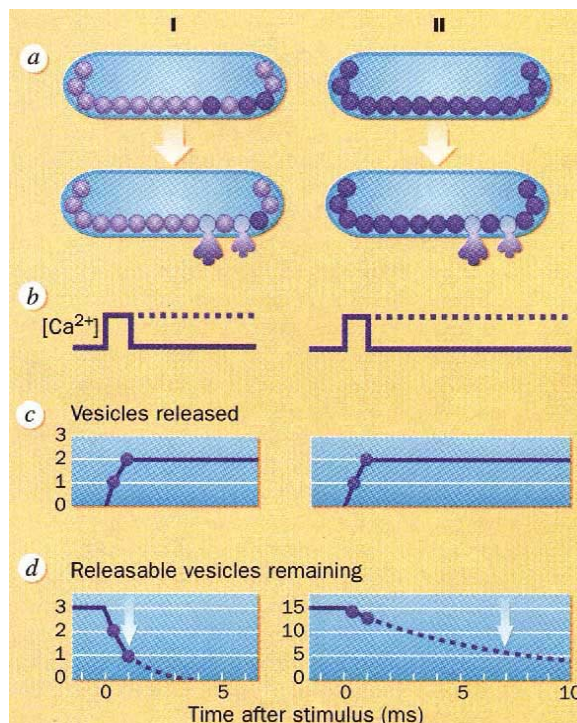
trated in the accompanying box. An 'exocytic time' is defined by asking the following question — once Ca has bound to the Ca sensor, how long does the exocytic machinery need *on average* to accomplish transmitter release? With release occurring in under 1 ms, most of us have concluded that the exocytic time is similarly short. That conclusion follows only if a sizeable portion of the releasable vesicles is, in fact, released by one action potential. And that, in turn, implies that only very few of the vesicles docked at release zones — maybe only one — are readily releasable (see box, model I). For several synapses, this view is supported by analyses of fluctuations in transmitter release during successive stimuli^{8,9}. An exocytic time in the millisecond range would generally accommodate only one fast biochemical reaction. Most of the rich synaptic biochemistry discovered over the past few years¹⁰⁻¹² would have to happen before the action potential arrived at the terminal.

However, if it were not for the statistical findings, fast transmitter release would also be consistent with much longer exocytic times. In model II (see box) the vesicles released are assumed to be drawn from a large pool. Rather than an intrinsi-

Synaptic transmission: high speed with slow molecules?

LARGE synaptic terminals release vesicles from tens to hundreds of release zones, each with dozens of vesicles. As an example, *a* in the figure shows two release zones, I and II, each with 15 synaptic vesicles docked beneath the plasma membrane, and each before and after an action potential. In II, all 15 vesicles can respond equally to Ca with exocytosis. In I, only three of the 15 vesicles can readily do so, while the remainder are in a dormant state. Assume that an action potential raises the local $[Ca^{2+}]$ next to each vesicle to saturating levels for a period of 1 ms (*b*). In response, each terminal releases two vesicles (*c*).

Although both terminals achieve the same result, terminal I requires a faster exocytic machinery than terminal II, releasing as many vesicles per millisecond while drawing on a five-times smaller pool. In I, the pool of releasable vesicles is two-thirds exhausted within 1 ms of the advent of high $[Ca^{2+}]$, while in II exhaustion is only 10 per cent. To make this more precise, assume that a single reaction is rate-limiting in exocytosis, and that $[Ca^{2+}]$ remains high indefinitely (dashed



in *b*). In this case (*d*) exocytosis exhausts the pool of releasable vesicles along an exponential function (dashed), with $1/e$ of the vesicles remaining after one time constant, τ (arrows in *d*). τ also equals the 'exocytic time', defined here as the

mean time for an exocytosis-competent vesicle to perform exocytosis at saturating $[Ca^{2+}]$. It is 7 ms in II and 0.9 ms in I, hence the exocytic machinery is seven times faster in I. To achieve speed, II relies on a rapid rise and fall of $[Ca^{2+}]$, on a large number of vesicles and on statistics to ensure that a lucky few have enough thermal energy to complete exocytosis ahead of schedule.

The validity of these two models can be tested by observing fluctuations in transmitter release from stimulus to stimulus and measuring their variance. Model II predicts that the variance equals the mean number of vesicles released, model I that it is less. Experiments favour model I. The figure also suggests that the exocytic time can be measured directly by forcing a maintained step increase in cytosolic $[Ca^{2+}]$, by observing the exhaustion of rapidly releasable vesicles during exocytosis and by measuring the time constant of exhaustion. In neuroendocrine cells secreting dense core granules, such measurements indicate an exocytic time of 40 ms at room temperature¹³. W. A.

cally fast exocytic machinery, the speed of release would then reflect fast regulation by the rapid rise and fall in $[Ca^{2+}]$. Von Gersdorff and Matthews's results tend to support the second view. When Ca channels were kept open with a voltage step, the cell surface increased rapidly at first, suggesting release rates approaching those inferred to occur after the arrival of an action potential. But after 200 ms, the increase in cell surface stopped, even though Ca flux continued and the local $[Ca^{2+}]$ presumably remained high. Evidently a large pool of vesicles (more than 33 in each release zone) became exhausted with a surprisingly slow time constant of 124 ms, three times longer than the exocytic time for dense core granules in pituitary cells¹³. A lot of biochemical events could take place in such time.

Must we now find an alternative explanation for the statistics of transmitter release? Is the exocytic time really 124 ms? The authors cautiously avoid this conclusion. Future experiments could explore whether faster time constants are obtained with higher cytosolic $[Ca^{2+}]$, and whether smaller, more rapidly exhausted pools of vesicles went undetected. Regardless of the outcome, the approach taken by von Gersdorff and Matthews is extremely promising. It is now also being applied to sensory hair cells of the inner ear¹⁴, another cell that releases synaptic vesicles. The method will ultimately allow the direct measurement of reaction rate constants while microinjected peptides, antibodies and antisense messenger RNAs modify the functions of proteins required in exocytosis. In the months to come, such studies will give tantalizing insights into the final steps in transmitter release. □

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Two-faced mimicry

Jared M. Diamond

BIOLOGICAL mimicry typically involves a cast of three characters: a mimic, which resembles another species and thereby gains some advantage for itself; a model, which is imitated by the mimic; and an audience, which mistakes the mimic for the model and thereby becomes the victim of the mimic's deception. L. Spear and D. Ainley (*Auk* **110**, 222–233; 1993) have just described a case of two-faced mimicry, in which the mimic gains distinct types of advantage from two different audiences, one of which is the model itself. These observations help clarify the most famous example of visual mimicry among birds, a complex and puzzling case described 125 years ago by Alfred Russel Wallace (*The Malay Archipelago*, Vol. 1, 150–153; Macmillan, London, 1869).

The models recognized by Spear and Ainley are large skuas, seabirds which are loathed by viewers of natural history programmes for their habit of killing baby penguins at nesting colonies. At sea, skuas obtain food mainly by 'kleptoparasitism', meaning that they search for smaller seabirds that have caught food, chase them, and force them to drop or regurgitate the food. If the victim resists, the skua pushes or knocks its victim out of the air into the water, bites it, shakes it by the neck, or holds its head under water. As a result of these terror

tactics, many victims do not even attempt to flee or to hold their catch but give it up as soon as they see a skua closing in.

The skuas' mimic is the Kermadec petrel, whose relatives constitute skuas' favoured victims. This particular petrel resembles the largest skuas not only in profile but also in sharing the skuas' conspicuous white wing patch, visible from afar. Skuas avoid attacking other skuas, presumably because the intended victim would be too dangerous. This suggests that the mimic gains one advantage by deceiving the model itself.

How can one be sure that the Kermadec petrel's immunity is due to mimicry? Naturally, we cannot ask skuas to explain the reasons for their unique charity to-

wards this otherwise tempting target so similar to them in profile, wing pattern and flight. However, it is striking that not one of the hundreds of Kermadec petrels observed by Spear and Ainley was attacked by large skuas, at the same time that the skuas were making 91 at-



Three examples of mimicry of *Philemon* friarbirds (left in each case) by *Oriolus* orioles. The islands concerned are Ceram (top), Buru (middle) and Wetar (bottom). The illustration is a detail from a painting by H. Lee Jones.

tacks on the seven other species of similar-sized petrels observed (such as Juan Fernandez petrels and wedge-tailed shearwaters). From the numbers of individuals of each species encountered, one calculates that the zero attack rate on Kermadec petrels is significantly lower ($P = 0.037$) than the attack rate on its relatives.

As to the other advantage gained by Kermadec petrels through mimicry, Spear and Ainley recorded 16 cases in which Kermadec petrels turned the tables and forced seabirds (mainly other petrels) of equal or even slightly larger size to give up food. Unlike most petrels, which fly by long glides interspersed with infrequent flaps, Kermadec petrels approaching a

victim imitate skuas' flight behaviour: they suddenly accelerate to high speed by continuously flapping their wings. Half of the victims released their catch immediately, even though the Kermadec petrel could hardly have beaten up the victim physically as can the two largest skuas (respectively 1.7 and 3.4 times heavier than the petrel). Thus, the victims were probably mistaking the Kermadec petrel for the much more dangerous skuas. In short, by its two-faced mimicry the Kermadec petrel deceives one audience into not attacking it, while deceiving the other audience into not resisting its own attacks.

The earliest description of avian visual mimicry was Wallace's account of another case involving large aggressive models and smaller mimics that would otherwise have been expected to be among the models' victims. The models are friarbirds of the *Philemon [moluccensis]* superspecies, which are among the largest members of a family (Meliphagidae or honey-eaters) notorious for pugnacious behaviour; the models are orioles of the *Oriolus [bouroensis]* superspecies (family Oriolidae). Wallace was struck by parallel geographical variation in plumage between friarbirds and orioles on two Indonesian islands. Subsequent study expanded Wallace's observations in three respects (E. Stresemann *Novitates Zoologicae* 21, 25–153, 1914; J. Diamond *Auk* 99, 187–196, 1982).

First, the parallel variation extends to seven islands, including New Guinea (see illustration for three examples). That is, friarbirds vary greatly in plumage among the seven islands, and so do orioles, but on each island the friarbird and oriole are very similar. Second, on each island the closeness of plumage mimicry is inversely (and paradoxically) related to closeness in size: the larger the friarbird's size advantage (hence the more dissimilar the model and mimic are in size), the more closely the oriole resembles it in plumage. Several smaller friarbird species on the same islands are not models, nor is a larger oriole related to the familiar gold-and-black European oriole a mimic. Finally, on New Guinea the oriole is in turn mimicked by the smaller streak-headed honey-eater *Pycnopygius stictocephalus*.

All of these species belong to a guild of aggressive birds that gather in flowering and fruiting trees, and establish a size hierarchy whereby larger birds drive off smaller birds. However, the mimic streak-headed honey-eater is never attacked by its oriole model, which in turn is never attacked by its friarbird model. Spear and Ainley's results warn us that the two mimics may derive an extra advantage from being able more easily to intimidate rivals as well as aggressors.

Many other cases of small mimics and large aggressive models have now been

reported, among birds as diverse as birds of paradise, African drongos and bush-shrikes and flycatchers, and neotropical toucans, cotingids and tyrant flycatchers. The puzzling mammalian example of the similar appearances of meek little insect-eating aardwolves and ferocious big lion-killing hyenas also comes to

mind. All these cases now warrant re-examination as further possible examples of two-faced mimicry. □

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MILLISECOND PULSARS

The times they are a-changing

S. E. Thorsett

THESE are boom times for the study of recycled radio pulsars, and not only because of the 1993 Nobel Prize in Physics awarded to Russell Hulse and Joe Taylor for their discovery of the first binary pulsar. A new generation of sensitive surveys aimed at high galactic latitudes and globular clusters is turning up these pulsars at a remarkable rate: the most recent pulsar catalogue¹, already outdated, lists 29 binary pulsars and a dozen more that were probably spun-up by mass transfer from a companion that has since been destroyed or lost. In aggregate, these pulsars have become a sample large enough for meaningful statistical investigations, while individually they show a rich variety of fascinating astrophysics.

If pulsar astronomers needed any further excuse for a January gathering beneath the ski slopes of Aspen*, it was provided by a belated celebration of the tenth anniversary of the discovery² of the first, and fastest, millisecond pulsar. Familiar theoretical problems — including the limiting spin period of neutron stars, whether neutron stars can form through the accretion-induced collapse of white dwarfs, how pulsar field strengths evolve, and how high-energy radiation is produced in pulsar magnetospheres — provoked lively discussion. But for me, the real excitement came from the rapid pace at which new and often unexpected observations continue to drive the field.

The hunt for fainter, faster pulsars now makes great demands on time at most of the largest radio telescopes. A team working at the Parkes telescope in Australia (R. N. Manchester, CSIRO) is surveying the entire sky south of the Equator, using nearly three months of telescope time. With about 65 per cent of their observations completely reduced, they have turned up 78 new pulsars. Ten of these have periods below 16 milliseconds, and seven of those ten are in binary systems. Together with new results from other observatories (R. S. Foster, Naval Research Laboratory; A. G. Lyne, University of Manchester; D. J. Nice,

National Radio Astronomy Observatory), this brings the known number of millisecond pulsars in the galactic plane to 25; the inferred number of millisecond pulsars in the Galaxy is between about 25,000 and 42,000 (M. Bailes, CSIRO), assuming a lower luminosity cut-off of 2.5 mJy kpc². This estimate is several times lower than believed a few years ago^{3,4}, reflecting better statistics and improved pulsar distance estimates, and helps to reconcile a long-standing discrepancy between the birth rates of millisecond pulsars and of low-mass X-ray binaries, their putative progenitors.

A typical binary pulsar is old, with a degenerate companion that is the remnant of the mass-transferring star from an X-ray binary phase. But two pulsars have instead been found to be orbiting massive main-sequence stars and thus appear to be ancestors, rather than descendants, of X-ray binaries. One of these, pulsar B1259–63, is in a high-eccentricity, 3.4-year orbit around a Be star⁵. Coincidentally, its latest periastron passage occurred on 9 January 1994, just after the end of the Aspen meeting. Observations were made with several X-ray satellites around periastron, looking for evidence of accretion onto the pulsar or the formation of a bow shock as the pulsar ploughed through the companion's dense stellar wind. The radio observations available by the meeting were already tantalizing (Manchester). As the pulsar plunged towards periastron in early December, the radio emission, which is ordinarily nearly 100 per cent linearly polarized, was depolarized and absorbed, disappearing at low frequencies about a month before periastron and at progressively higher frequencies in the next few weeks. Together with observations of the egress, presumably in late January, these measurements will provide good constraints on the density and magnetic field strengths in the Be wind.

No such wind is observed around the B star companion of pulsar J0045–73, in the Small Magellanic Cloud (V. M. Kaspi, Princeton University). This pulsar, in a highly eccentric 51-day orbit, shows no evidence of regular eclipses or extra dis-

* 1994 Aspen Winter Conference on Astrophysics, Millisecond Pulsars: A Decade of Surprise, Aspen, Colorado, 3–8 January 1994.

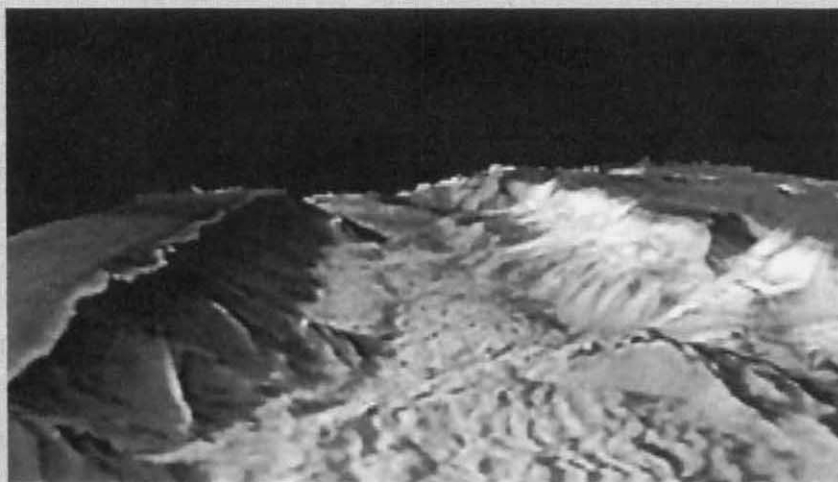
persive delay; indeed, the wind density implied is lower than that expected for an isolated B star. The spectral type and photometry indicate that the companion mass is greater than 10 solar masses, and optical spectroscopy may allow a test of this conclusion.

These two companions are the most massive objects found orbiting radio pulsars. Also announced in Aspen (A. Wolszczan, Pennsylvania State University) was the lightest: a Moon-sized body in a 25-day orbit around the pulsar B1257+12, a pulsar that had previously been deduced to have two planets a few times bigger than the Earth⁶. Wolszczan also laid to rest any serious doubt that the original double-oscillatory period variations observed in this pulsar are gravitationally induced by orbiting planets, by announcing the detection of the predicted secular variations of the bodies' orbital parameters due to their mutual perturbations⁷.

Another intriguing candidate for a pulsar-planetary system came in the form of quasi-oscillatory phase variations of the slow pulsar B1828-11 (Lyne), which might be consistent with free precession of the neutron star⁸, but which can also be decomposed into the sum of three keplerian orbits.

Indirect evidence for a planet orbiting the binary pulsar B1620-26, in the globular cluster M4, came last year from an anomalously large second-derivative of the period^{9,10}. Measurements of a period third-derivative (D. C. Backer, University of California, Berkeley, and myself) give a characteristic orbital timescale (for a circular orbit) of 160 years, and an implied mass of 80 times that of the Earth. However, when eccentric orbits are considered (F. C. Michel, Rice University; S. Sigurdsson, University of California, San Diego) the allowed companion mass rises to a solar mass or more. Consideration of dynamical instability and secular perturbation of the orbit of the inner body by that of the outer leads most naturally to a stellar-mass companion, with an orbital period measured in centuries (F. A. Rasio, Institute for Advanced Studies, Princeton). Such triple systems are naturally expected in the dense cores of globu-

A new angle on Mars



LOOKING at this image of the surface of Mars, one has the eerie impression of seeing the landscape from the window of an aeroplane. How is it possible to survey the planet this way?

The answer lies in stereo-pair images taken from the Viking Orbiter satellite and processed by G. Thornhill and co-workers (*J. geophys. Res.* 98, 23581-23587; 1993). Software developed at University College London automatically matches several thousand points, making light work of what would previously have been months of tedious manual labour. As the images are taken with different geometries, the two different relative positions of each point can be used to derive its height provided one knows the spacecraft's elevation and the camera direction.

The result is a grid of known heights (the obligatory acronym is a DEM, or digital elevation model). One image from the stereo pair is combined with this grid on the computer — essentially, the original photograph provides a pattern of light and shade to be draped

over a scaffolding of heights.

A perspective view can be generated from any apparent height and direction above the surface. This particular image is the view along Tithonium Chasma, part of the great Valles Marineris chasm system, as it would appear from 30 kilometres up. The chasm is about 7 kilometres deep and 40 kilometres wide at this point, putting Colorado's Grand Canyon, a mere 1.9 kilometres deep, in the shade.

In the immediate foreground, part of the chasm wall has slumped in a landslide, spreading in folds and corrugations almost to the opposite wall of the channel. Those whose taste is more for huge impact craters or putative dried beds of ancient waterways will find them in the original paper.

Impressive as their images are, Thornhill and colleagues strike a mournful note in their introduction. If the Mars Observer were still with us, direct measurements of the martian topography from laser altimeter data could have been flooding in. L. M.

lar clusters, but the expected lifetime is short compared with that of the millisecond pulsar.

Caution is needed, though, in interpreting such timing irregularities. Systematic deviations from a simple spin-down model have also now been observed, albeit at a much lower level, in eight years of observations of the original millisecond pulsar B1937+21 (Kaspi). Because similar instabilities are not seen in comparable data for pulsar B1855+09 (an older pulsar, but discovered more recently), and multi-frequency observations seem to rule out propagation effects as the cause, the simplest conclusion is that intrinsic rotational instabilities are being observed. Although 'timing noise' is familiar in young, slow pulsars, this is the first time it has been observed in a recycled pulsar.

So a decade after its discovery, the natural limits of the precision of one of nature's most remarkable clocks are finally being explored; by coincidence, its stability, slightly better than one part in 10^{14} in a few years, is comparable to the best terrestrial atomic clocks. We now know that B1937+21 is young for a millisecond pulsar, however, and already observations of pulsar B1855+09 suggest it may reach stabilities of a few parts in 10^{15} or better. As such pulsars are discovered and studied, pulsar astronomers have good reason to expect that the next decade will prove as exciting as the last. □

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Hot lips and phosphorylation of protein kinases

C. J. Marshall

ONE of the successes of the study of signal transduction has been the elucidation of several 'MAP kinase' pathways. These pathways involve the sequential activation of protein kinases, and they control diverse cellular responses in eukaryotes including proliferation and differentiation¹.

Another milestone has now been reached with the publication by Zhang *et al.*, on page 704 of this issue², of the crystal structure of the mammalian MAP kinase

One end result of the activation of MAP kinases is their ability to phosphorylate and thereby activate a number of transcription factors, a process which may hinge on their ability to enter the nucleus. A function of MAP kinases may therefore be to provide a link between transmembrane signalling and the nucleus. The biochemical logic of having a MAP kinase regulated through threonine and tyrosine phosphorylation by a dual specificity MAP kinase kinase, which is itself acti-

DFGFAKRVKGR...WTLCGTPEYLAPE	cAPK
DFGLARIADPEHDHTGFLTEYVATRWRAPPE	ERK2 (ref. 6)
DFGLARAFGVPRTY...THEVVTLWYRAPE	CDK2
DFGFAKOLRAENGLL...MTPCYTANFVAPE	RSK (ref. 12)

Activating phosphorylation sites in protein kinases (single-letter amino-acid code). The activating phosphorylation sites are highlighted.

ERK2. This is only the third protein kinase structure to have been solved, the first being the catalytic subunit of cyclic AMP-dependent kinase (cAPK)^{3,4}, and the second cyclin-dependent kinase 2 (CDK2)⁵. Overall, the organization of the three kinases is similar — all have a bilobate structure in which ATP is bound in the cleft between the two lobes and the carboxy-terminal lobe binds the substrate. But the newly determined structure is in some ways very different from those of cAPK and CDK2, providing insight into substrate specificity and the way in which the activity of MAP kinases is regulated by phosphorylation.

MAP kinases were first discovered in mammalian cells as cytoplasmic protein kinases whose activity is rapidly stimulated by growth factors. Their distinguishing characteristic is that they are activated by phosphorylation on threonine and tyrosine residues within the motif Thr-Glu-Tyr⁶, a process which is carried out by a dual specificity protein kinase, MAP kinase kinase⁷ (known, in mammalian cells, as MEK).

A mechanism of MEK activation is through phosphorylation by the serine/threonine protein kinase Raf; Raf itself is activated following the stimulation of receptor tyrosine kinases, by a mechanism which involves the formation of Ras/GTP and binding of that complex to the Raf kinase⁸. Similar signalling pathways from receptor tyrosine kinases have been shown to operate in the nematode *Caenorhabditis elegans* and the fruitfly *Drosophila melanogaster*.

vated by serine/threonine phosphorylation by MAP kinase kinase kinases, is not only used by multicellular organisms but also by single-cell eukaryotes. In the yeasts, for example, MAP kinase pathways are required for the maintenance of cell shape, osmotic integrity and the responses to mating pheromones. Each of these pathways has its own MAP kinase(s), with distinct MAP kinase kinases and upstream kinases⁹.

Since the discovery that MAP kinases are regulated by threonine and tyrosine phosphorylation, a central question has been how these phosphorylation events turn on the catalytic activity. The crystal structure of ERK2 is a great help in understanding this mechanism, for it suggests why dual phosphorylation is required to activate ERK2. The threonine (Thr 183) and tyrosine (Tyr 185) residues, phosphorylation of which by MEK activates ERK2, are contained in a loop (L₁₂) which connects the protein kinase domains VII and VIII; these domains include residues Asp-Phe-Gly and Pro-Glu essential to the catalytic activity of all protein kinases¹⁰. The catalytic activities of cAPK and CDK2 also require phosphorylation of a threonine residue contained within this loop, but Zhang *et al.*² show that the structure of the L₁₂ loop in ERK2 is quite different. In ERK2, L₁₂ is longer (by six amino acids compared with cAPK, three compared to CDK2) and forms what the authors term the 'lip'. The Thr 183 is exposed on the surface, and is therefore likely to be accessible to MEK, but Tyr 185 is buried in a hydrophobic

pocket. The location of Tyr 185 is perhaps the most striking feature of the structure and would not have been predicted by model building. To phosphorylate Tyr 185, MEK must presumably alter the conformation of the 'lip'; co-crystals of ERK2 and MEK are eagerly awaited.

The positioning of Tyr 185 in the inactive, nonphosphorylated form of ERK2 suggests that the 'lip' may occlude the substrate-binding site. On phosphorylation, Tyr 185 probably changes the position of its side- and main-chains so that substrate can now bind. If phosphorylation of Tyr 185 regulates substrate binding, what is the role of phosphorylation of Thr 183? Unlike the inactive form of CDK2 (ref. 5), the structure of the bound ATP is not distorted in inactive ERK2. But comparison of the structure with the structure of active cAPK shows that the catalytic residues of inactive ERK2 are not aligned correctly for phosphotransfer, the ATP-binding cleft being more open than in active cAPK. Phosphorylation of Thr 183 is predicted to lead to rotation of the amino- and carboxy-terminal domains, so that the catalytic residues become correctly aligned. If the crystal structure of active ERK2 confirms these predictions, we will have a most elegant explanation of why the activation of MAP kinases requires a dual phosphorylation event.

Phosphorylation within the L₁₂ loop is emerging as a regulatory mechanism common to many kinases. Activation of cyclin-dependent kinases requires phosphorylation by a cyclin-activating kinase at a threonine in this region (Thr 167 in *Schizosaccharomyces pombe* CDC2, Thr 161 in human CDC2 and Thr 160 in human CDK2), as well as interaction with a cyclin. Within the mammalian MAP kinase cascade, both the upstream MEK (ref. 11) and downstream RSK (ref. 12) are activated by phosphorylation in L₁₂ (see figure). So the L₁₂ loop and its 'lips' are likely to be happy hunting grounds for those interested in the regulation of protein kinase activity. □

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Hearing through a glass brightly

Lawrence Challis and Stanley Clough

To many, efforts to increase the efficiency with which sound can be amplified must seem a thoroughly antisocial activity. But this, of course, is audible sound where there is a well-tried route using a microphone to convert sound into electrical signals which are amplified and converted back to sound via loudspeakers. Ultrasound, at much higher frequencies, is extensively used in telecommunications, medical imaging and nondestructive testing; it is also a valuable probe for research on condensed matter, much of it carried out at low temperatures. Amplification at these frequencies of around 100 MHz and above is again normally electronic, with conversion to and from ultrasonic waves via transducers. This may sound familiar:

it was the approach originally used in optical communication to amplify, every 100 km or so, the light intensity passing down a glass fibre. But then, in 1987, Gambling and colleagues at Southampton University showed that optical amplification could be achieved within a fibre by doping the glass and pumping it to achieve population inversion. Prieur, Höhler, Joffrin and Devaud¹ have now shown that glass can also be made to amplify ultrasonic waves.

The first reports of ultrasonic amplification appeared in 1961. One technique, introduced by Tucker², achieved amplification at 1.5 K by inverting the populations of two levels of a Cr^{3+} ion in Al_2O_3 (ruby) separated by the energy of the

ultrasonic phonon, $\hbar\omega$. The ground state of this ion is split into a ladder of four separate levels (1 to 4, with 1 highest) by a combination of an external magnetic field with the electric field of the trigonal crystal. The magnetic field is tuned in magnitude and direction until levels 2 and 3 are split by the ultrasonic frequency of 12 GHz, and levels 1 and 3 (and 2 and 4) by a microwave pump frequency of 24 GHz. Before pumping, the population of level 2 is significantly less than that of 3. Pumping reverses this, because it increases the population of 2 while reducing that of 3. Ultrasonic amplification now takes place by stimulated emission as in a laser.

The levels couple to both ultrasound and microwave radiation, which is not too surprising, as the ultrasound makes the positive nuclei vibrate and this creates oscillating electric fields. In fact, the same system had been developed earlier as a microwave amplifier or maser, and was

Population inversion in the SASER

THE aim of adiabatic rapid passage (ARP) is to exchange the populations of two levels separated by the energy $\hbar\omega_0$ by sweeping the frequency ω of an oscillating disturbance (the acoustic strain in the present case) through ω_0 . When $\omega = \omega_0$ the amplitudes p and q , from which the populations $|p|^2$ and $|q|^2$ of the two levels are obtained, oscillate with a frequency ω_1 which depends on the amplitude of the strain and its coupling to the TLS. More generally

$$dp/dt = -i\omega_0 p/2 - i\omega_1 \exp(-i\omega t)q$$

$$dq/dt = i\omega_0 q/2 - i\omega_1 \exp(i\omega t)p$$

By choosing two new variables p' and q' the equations are transformed into ones of the form $dp'/dt = i\Omega p'/2$ from which the disturbance has disappeared because it has been incorporated coherently into the TLS. The energy levels of this composite TLS are $\pm \hbar\Omega/2$ where $\Omega = ((\omega_0 - \omega)^2 + \omega_1^2)^{1/2}$, and show 'anticrossing' behaviour (see Fig. 1). As ω changes through ω_0 the eigenstates of the composite TLS change between the eigenstates of the original TLS, and the effect is to exchange the latter's populations. The technique requires the change of ω to be slow enough for the state of the composite TLS to follow, but if the change is

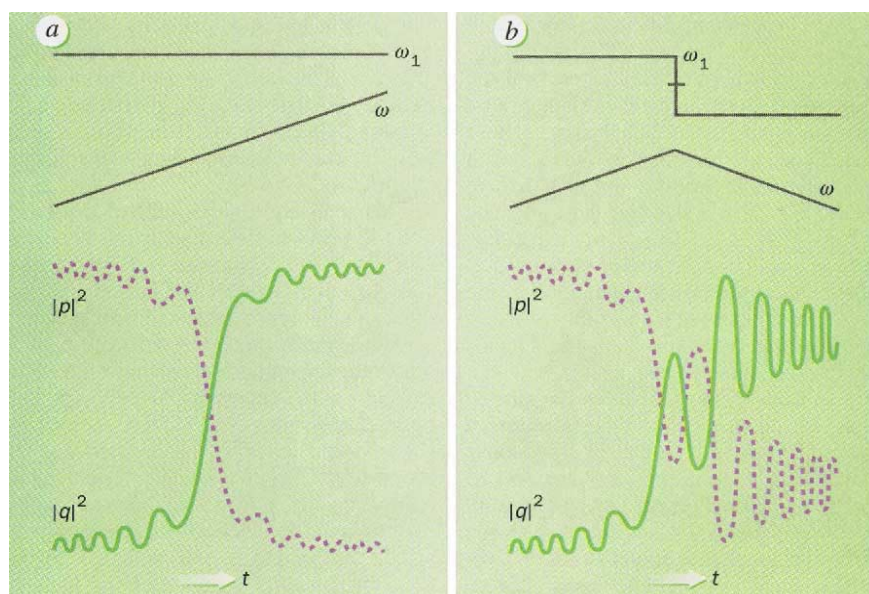


FIG. 2 Simulations of the usual ARP (a) and the variant (b) in which the direction of sweep is reversed at ω_0 with a phase switch. The sweep speed is as large as can be tolerated while achieving a reasonable degree of inversion.

too slow the population inversion decays because of relaxation processes. A compromise is necessary. Oscillations of the amplitudes p and q are generated and the

inversion is less than perfect.

Prieur and colleagues' acoustic amplifier uses an unusual variant of ARP in which ω is swept to ω_0 and back again with a sudden switch of phase of π where the direction of the sweep is reversed. The usual ARP follows the two paths in Fig. 1a. The route of the variant is shown in Fig. 1b. Figure 2 shows simulations of standard ARP and the variant under conditions of compromise between sweep rate and inversion efficiency. The variant only inverts populations for TLS having ω_0 close to the extreme value of ω . Standard ARP would invert populations over a wider band of TLS frequencies.

L.C. & S.C.

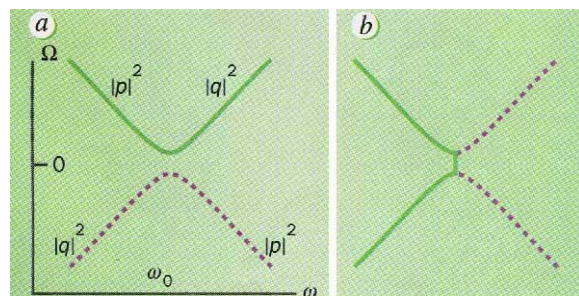


FIG. 1 The levels of the composite TLS exhibit anticrossing which is exploited in ARP. a, Usually $(\omega - \omega_0)$ is swept through 0 following the paths shown. b, The variant reverses the direction of sweep at 0.

used in the first transatlantic satellite broadcast.

The second technique, first reported just a few months later, uses the interaction of ultrasonic waves with the conduction electrons in a semiconductor. Normally there is a net loss of ultrasonic energy into Joule heat produced by the currents generated by the ultrasonic wave. But if, by applying a d.c. electric field, you force the electrons to drift with a velocity greater than the velocity of sound v_s , they emit phonons, a process akin to Cerenkov radiation. An electron moving through the lattice attracts atoms towards its path and so leaves behind a vibrating wake — a shower of phonons covering a wide range of frequencies. Each is the result of an electron making a transition to a lower energy state which is normally a spontaneous process. But it can be stimulated by ultrasound of the same frequency, leading to ultrasonic amplification.

This process was demonstrated at 15 and 45 MHz in a CdS rod at room temperature by Hutson, McFee and White³. As predicted, ultrasonic attenuation switched to amplification when the drift velocity reached v_s . (The technique was later shown by others to work even better when both electric and magnetic fields were applied.) Since then, these acoustoelectric techniques have been much more widely used to amplify surface acoustic waves in a strongly piezoelectric material such as LiNbO₃. This is insulating, so to obtain amplification the material is placed in contact with a semiconducting slab or layer carrying a rapidly drifting current of electrons to which the surface wave can couple.

Prieur and colleagues¹ have developed an interesting variation on the population inversion approach used in the early 1960s. Their amplifier is a 2-cm rod of pure silica glass 'Tetrasil' which, like all amorphous materials, contains a large number of localized defects called 'two-level systems' (TLS). The level separations in these systems are known to cover a wide spectrum and their very strong coupling to ultrasonic waves normally attenuates the wave as it passes through the glass.

Prieur and colleagues have demonstrated, however, that the wave can be amplified if population inversion is produced in those two-level systems whose level separation matches the ultrasonic frequency. Since the systems only have two levels, inversion cannot be produced by the pumping technique used for ruby which depends on differential relaxation rates within a set of levels. So they adapted an NMR technique called adiabatic rapid passage (see box on previous page) to transfer coherently the populations of the lower and upper levels using an ultrasonic pump.

The gain achieved in these first experi-

ments is not impressive — just 2 dB at 20 mK for 340 MHz waves — but should increase with frequency. The authors also envisage increasing the gain by using an acoustic cavity to produce a sound laser: this, naturally, they dub a 'SASER'. The system has the great benefit that the amplifier can be tuned over the very wide range of frequencies covered by the level separation of the two-level systems as in a dye laser.

It seems likely that the need to work at low temperatures, $kT \lesssim \hbar\omega$, will restrict this approach to the research laboratory.

Even so, it is a development not to be sneezed at; if the gain can somehow be improved, it should have real benefits in extending the range of possible ultrasonic experiments. □

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PROTEIN–NUCLEIC-ACID INTERACTION

A molecular handshake

Dinshaw J. Patel

STRUCTURAL studies of the recognition of the double helix by DNA-binding proteins have provided details of the intermolecular interactions that account for the sequence specificity of the binding process¹. The DNA-binding proteins studied to date complex to both the major and minor grooves of the helix, the most pronounced helical distortions being associated with bends and kinks that occur without disruption of the Watson–Crick base-pairing alignments².

This seeming inviolability of the DNA helix is now shattered with the report in *Cell*³ of the structure of a ternary cofactor–protein–DNA complex that mediates the methylation of the cytosine 5 position. Both the DNA and the protein undergo dramatic conformational transitions, with segments of the protein penetrating into the DNA helix and occupying the cavity generated by the looped-out target cytosine. The ternary complex is of additional interest as the protein and the DNA are covalently cross-linked and include the cofactor in the reaction product state. Cheng, Roberts and colleagues' remarkable results^{3,4} have emerged from crystallographic studies of M.HhaI cytosine-5-methyltransferase (m5C-MTase) in a binary complex with

S-adenosyl-L-methionine (AdoMet)⁴, and in a ternary complex with the d(G-C-G-C) cytosine methylation site (designated *C) and the reaction product S-adenosyl-L-homocysteine (AdoHcy)³.

The M.HhaI from *Haemophilus haemolyticus* is a m5C-MTase which recognizes the d(G-C-G-C) duplex segment and catalytically transfers a methyl group from AdoMet to the first cytosine in this sequence. This reaction pathway^{5,6} involves a key covalent intermediate (2 in Fig. 1) that results from nucleophilic attack at the cytosine C6 position by the conserved Cys 81 residue on the m5C-MTase. The intermediate can be trapped following replacement of the proton by a fluorine at the cytosine C5 position⁷. This strategy was employed to generate the ternary complex of covalently linked m5C-MTase and DNA along with the cofactor for the crystallization studies³.

The crystal structure of M.HhaI m5C-MTase in the presence of AdoMet was solved last year, at 2.5 Å resolution⁴. The enzyme has two domains, separated by a linker segment, whose relative alignments generate a cleft that was predicted to be the DNA-binding site. Both the AdoMet cofactor binding site and the essential catalytic Cys 81 residue are located on the

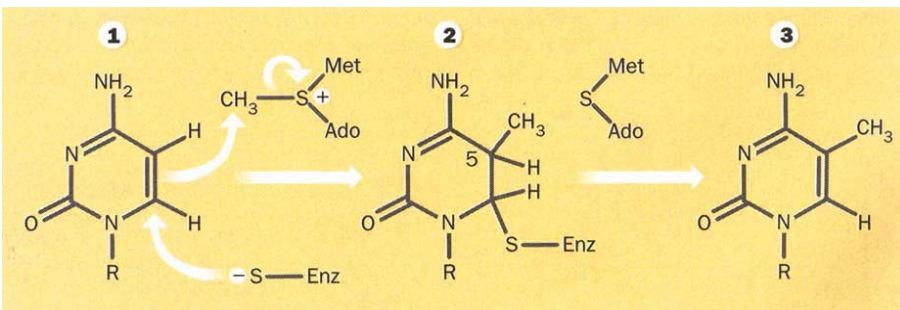


FIG. 1 Proposed reaction pathway for methylation by AdoMet at the cytosine 5 position on DNA in the reaction catalysed by m5C-MTase. The covalent m5C-MTase–DNA adduct 2 for the crystallization study of the ternary complex was trapped by replacing the proton at the cytosine 5 position by a fluorine. (Figures 1, 2 and 3 courtesy X. Cheng, ref. 3.)

RÉSUMÉ

Whirls apart

THE feeling of sitting in the midst of chaos is not an uncommon one. But, say Richard L. Kautz and Bret M. Huggard, anyone who really wants to experience chaos should try the nearest amusement park (*Am. J. Phys.* **62**, 59–66; 1994). Their subject is a fiendish ride known as the 'Tilt-a-Whirl' with seven cars each spinning freely about the centre of its own platform, hurled around an undulating track by radial arms. Kautz and Huggard's mathematical description of the ride shows it to be a fine example of deterministic chaos, characterized by apparently random dynamics and extreme sensitivity to initial conditions. Shifting position in the car, for instance, can turn a tame ascent into a frenzied whirl. Not recommended reading for the queasy.

Gutter press

THERE'S no need to scour the Amazon jungles or the depths of the sea in search of species as yet innocent of scientific description — R. Bertolani and I. M. Kinchin found theirs in the sediment clogging a rain gutter in Guildford, as remote a place as any within a short train ride from London. Bertolani and Kinchin were looking for tardigrades (water bears), microscopic creatures thought to be akin to arthropods. Among five species recovered, one was distinguishable from known tardigrades by its ornate egg-shell architecture in particular, and it now rejoices in the name *Ramazzottius varieornatus* (*Zool. J. Linn. Soc., Lond.* **109**, 327–333; 1993).

Piece work

As all children know, one way to find out how something works is to take it to pieces and try the components in different combinations. Such is the tack taken by H. Stenmark *et al.* (*EMBO J.* **13**, 575–583; 1994), who have created hybrids of two members of the rab family of GTP-binding proteins. These proteins are found in distinct organelles in the cell, and each seems to have a highly specific job in directing particular vesicles to a particular destination. Stenmark *et al.* find that to make one of the rabs carry out the other's function not only did they have to transfer the C-terminal domain known to be important in targeting, but also the N terminus and two other domains involved in nucleotide-dependent conformational changes. Hybrids such as these will be of great help in identifying accessory proteins which interact with rabs and assist in taking vesicles to their correct destination.

Second thoughts

This year there will be a leap second on 30 June between 23:59:59 and 00:00:00 Universal Time, by decree of the International Earth Rotation Service (*IAU Circ. No. 5929*). Plan now to make the most of the extra time.

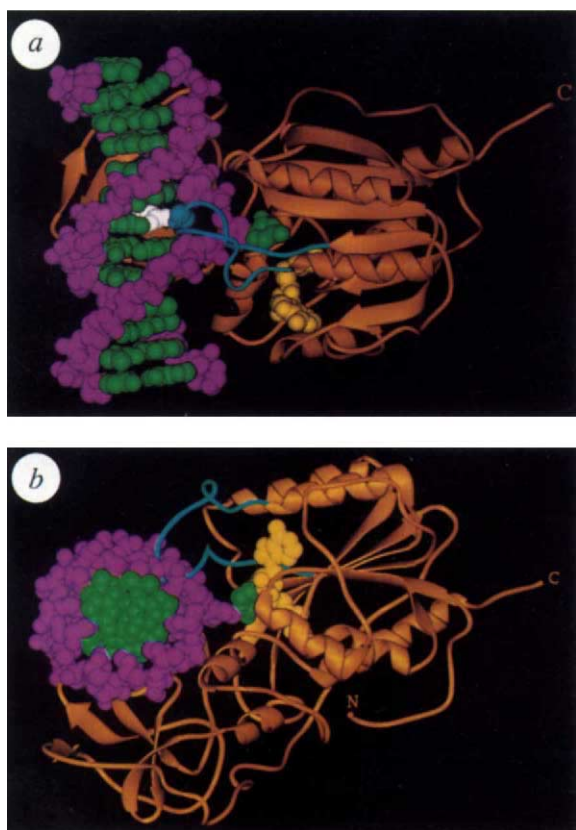


FIG. 2 Two views of the ternary complex formed by the M.HhaI m5C-MTase, a 13-mer DNA duplex containing its recognition sequence, and AdoHcy, the end product of the reaction. (DNA bases, green; sugar-phosphate backbones, magenta. Protein, brown; active-site loop and Ser 87, blue; Gln 237 on one of the two glycine-rich recognition loops, white. Cofactor AdoHcy, yellow). *a*, View normal to the helix axis, with the large domain on the right and the small domain positioned behind the DNA. *b*, View down the helix axis with the large domain on the right and the small domain below the helix. The DNA nestles in a cleft between the large and small domains.

large domain, as are most of the invariant residues which are positioned in the loop regions facing the cleft. The small domain contains the variable regions postulated to recognize the target sequence. The catalytic Cys 81 residue is in a loop region in the cleft adjacent to the AdoMet-binding pocket. However, the 10 Å separation between the donor methyl of AdoMet and the sulphur atom of Cys 81 in the binary complex suggested that a conformational transition must occur to bring these atoms closer together on formation of the ternary complex with DNA.

Now that we have a ternary structure³, with a resolution of 2.8 Å, what does it tell us? Two views of the complex appear in Fig. 2 and show its unique recognition features. The m5C-MTase interacts with the DNA using both the active-loop site on the large domain (residues 80–99; blue in Fig. 2) which contacts the minor groove, and two glycine-rich loops on the small domain (residues 233–240 and 250–257) which contact the major groove. This results in the d(G-f⁵C-G-C) binding site on the DNA duplex being enveloped by

represents a reaching out between extended segments on the enzyme and the DNA, and the resulting intermolecular interactions constitute a handshake at the molecular level.

The DNA oligomer in the ternary complex consists of a dodecanucleotide duplex with single-base overhangs, and adopts helical parameters characteristic of a B-DNA helix, with standard Watson-Crick base pairs, except for the segment centred about the target cytosine site. The dihydrocytosine intermediate (2 in Fig. 1) that results following Michel addition and methyl transfer adopts a C4'-*exo* sugar pucker and a *syn* glycosidic torsion angle. Both the base and sugar of the target cytosine are looped out of the helix, and this is accompanied by large distortions of the flanking phosphates that propagate in the 5' direction along the phosphodiester backbone. The resulting increase in the interstrand phosphorus-phosphorus distance provides a pathway for looping the cytosine out of the helix. The Cys 81 is covalently linked to the C6 position of the targeted cytosine, with the

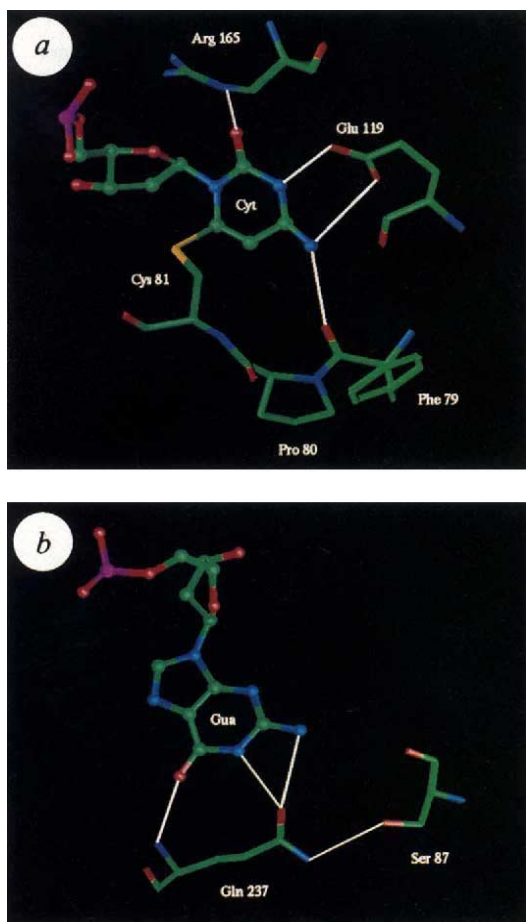


FIG. 3 Intermolecular hydrogen-bonding interactions involving the active-site cytosine and guanine residues in the ternary complex. *a*, The looped-out cytosine is covalently linked through its C6 position to the sulphhydryl of Cys 81, and is anchored through hydrogen bonds to its Watson-Crick pairing edge with the backbone and side-chain atoms of the invariant Phe 79, Glu 119 and Arg 165 residues. *b*, The Gln 237 side-chain on the recognition loop penetrates into the cavity generated by the looped-out cytosine and forms backbone and side-chain hydrogen bonds with the Watson-Crick pairing edge of the orphan guanine residue.

Cys 81 and AdoHcy positioned on opposite sides of the slightly nonplanar dihydrocytosine residue. The polar groups along the Watson-Crick pairing edge of the dihydrocytosine ring are all involved in intermolecular hydrogen bonds with invariant residues of the m5C-MTase in the complex (Fig. 3*a*). These interactions that define substrate specificity anchor the dihydrocytosine in its binding pocket through pairing with both side-chain and backbone groups on the enzyme.

The cavity generated by the looped cytosine is occupied by Gln 237 (white in Fig. 2*a*) from the small-domain, glycine-rich recognition loop, and by Ser 87 (blue) from the large-domain, active-site loop, which penetrate into the DNA helix from opposite directions in a pincer movement. The polar groups along the Watson-Crick pairing edge of the orphan guanine are all involved in intermolecular hydrogen

bonds with the backbone and side-chain atoms of Gln 237, whose alignment is stabilized through hydrogen-bond formation with Ser 87 (Fig. 3*b*). Interestingly, neither Gln 237 nor Ser 87 are conserved residues in m5C-MTases, meaning that hydrophobic interactions must also contribute to these intermolecular alignments.

Quite different positions are occupied by the AdoMet cofactor in the binary complex and the AdoHcy cofactor product in the ternary DNA complex. The AdoHcy is tightly bound in its cofactor-binding pocket, the site being accessible to solvent. One can surmise a sequence of steps leading to ternary-complex formation involving initial binding of m5C-MTase to the DNA followed by subsequent binding of the cofactor.

The structure of the cofactor-m5C-MTase-DNA ternary complex discussed here defines one step (2 in Fig. 1) on the reaction pathway for cytosine C5 methylation on DNA. Other issues that need to be addressed include a structural dissection of the enzyme-DNA contacts in the initial binding event that accounts for the observed sequence-specific recognition (1 in Fig. 1), and elucidation of the pathway for the return of the methylated cytosine into the DNA helix (3 in Fig. 1). It should be possible to tackle these questions by choosing suitable cytosine and cofactor analogues, and by creating mutations in the enzyme that would trap the required intermediates for structural characterization.

Finally, we can expect that the opening of the DNA helix observed in the ternary complex in the m5C-MTase system will be but the first example of motifs in other protein-DNA complexes that involve helix disruption. Variants of this structural theme may well turn up in DNA complexes ranging from those formed by replication-origin-binding proteins to enzymes catalysing recombination events. □

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DAEDALUS

Fat and fancied

DIETING and jogging make millions miserable, most of them women. They claim to be in it for health and fitness; but what they really want, of course, is sexual desirability. For the Western world thinks that slim is sexy. Some people denounce this as culturally imposed 'sizeism'; but Daedalus claims it is genetic. He argues that we have evolved to be sexually attracted by people who, in current conditions, are most likely to rear successful offspring. In societies where food is limited, fat women tend to be fancied — their fat reserves should see them through the metabolic challenges of pregnancy and lactation. In rich societies, food isn't critical, so slim women are desirable — their slimness implies that they are young and not pregnant already. Earlier in this century, even the West was so poor that heavy figures were popular, and many people tried to put on weight.

How does this come about? After all, nobody can choose to be attracted by this or that style of figure. Many forms of instinctive and sexual behaviour, human and animal, are imprinted by some crucial experience which occurs during a brief time-window of susceptibility. Daedalus reckons that there must be a critical period, probably quite early in infancy, when a baby 'decides', in effect, whether it is growing up in an over-fed or a starving society. If starvation seems to loom, it will be imprinted to fancy the fuller figure; if not, its sexual ideal will be slim.

Modern Western clinics and baby-care organizations judge the health of a baby by the speed with which it gains weight. Babies are given as much food as possible all the time, so they inevitably get fat. At the same time, of course, they become imprinted with extreme slimness as their sexual ideal. Hence the universal tragic mismatch between desire and reality which is sizeism.

So Daedalus proposes the experiment of inserting a controlled period of near-starvation into the feeding regime of otherwise well-fed babies. The age of onset and the duration of this period will be varied; when the infants mature, the figures they find attractive will be noted. This will reveal the crucial period for imprinting size preference. Western baby-feeding schedules will then be able to starve the next generation for just this critical period, while happily overfeeding it all the rest of the time. The blissful result will be a society of fatties, all programmed to fancy other fatties. Sizeism will vanish; dieting and jogging will perish unmourned. Health and fitness will decline, of course, but nobody cares about that.

David Jones

Lactation in male fruit bats

SIR — Although male lactation is physiologically possible¹, it is isolated and rare. It has been observed in domesticated mammals^{2,3} and in humans^{4,5}, but it has not been reported in wild, free-ranging species. Here we report lactation by males in a population of Dayak fruit bats, *Dyacopterus spadiceus* (Chiroptera: Pteropodidae), in the Krau Game Reserve, Pahang, Malaysia.

In July and August 1992, we captured 18 *D. spadiceus* in mist nets 8–30 m above the ground in the subcanopy of a lowland rainforest at Kuala Lompat in the game reserve. Of the 13 males that were captured, 10 were judged to be mature based on fully-ossified wing joints and descended testes. Each of the mature males also had functional mammary glands from which small amounts of milk were ex-

pressed. Among the five females that we captured, three were mature, but milk could be expressed from only one in response to manual palpation.

Although some milk was expressed from each of the adult males, the amounts were relatively small. From two males captured in late August, only 4–6 μ l were obtained, compared to 350 μ l from the right mammary gland of a single adult female. The nipples of the males were smaller and less cornified than those of the females, suggesting little if any suckling activity by the young.

We examined histological sections of the mammary glands and testes of three males, and the mammary glands of two females (one of which was lactating), using light microscopy (Fig. 1). The secretory tissue of the lactating female (a) was thicker and better developed than in the non-lactating female (b). In the males, secretory tissue occupied a layer similar in total thickness to that of the non-lactating female. In two of the males, most alveoli were more distended than in the lactating female, ranging in diameter from 80–140 μ m (c). The distension of the alveoli, as well as the consistent presence of secretory material, suggests that milk may have accumulated in the alveoli as a result of not being suckled. In the third male that we examined, the alveoli were less distended, resembling those of the lactating female.

Large mammary ducts were conspicuous in males. These ducts contained secretory material and coursed centrally towards the nipple from the peripherally located alveolar tissue. Multiple lactiferous ducts entered the nipple and opened onto its surface (Fig. 2).

The histological appearance of the testes in the two males with the more distended alveoli was consistent with active spermatogenesis. All stages of developing germ cells were observed in the seminiferous tubules, and these tubules had patent lumina. The significance of testicular histology in the third male was uncertain, as germ cells in all developmental stages were present, but there were few seminiferous tubules with open lumina.

Male lactation may not occur in all populations of *D. spadiceus* or it may vary seasonally. For example, from another sample of 17 individuals netted in Septem-

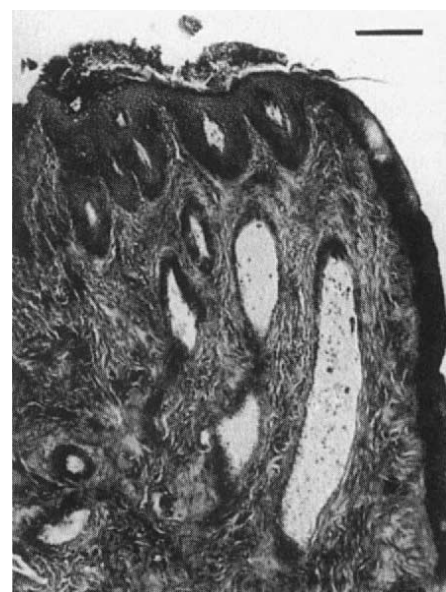


FIG. 2 Vertical section of the nipple of a lactating male *D. spadiceus*, illustrating lactiferous ducts. At the tip of the nipple (top), material expressed from these ducts appears to have adhered to the epithelium during fixation. Bar, 200 μ m.

ber 1992 in Sabah, six were mature males with enlarged testes, but none was obviously lactating, nor were the eight apparently mature females. In contrast, one of four mature males caught in August 1993 in Perak was lactating, as was one of the five mature females captured at the same time (two females were pregnant).

Male lactation has been reported only in highly inbred domestic animals^{2,3} or in humans in association with hormone treatments or pathological conditions^{4,5}. Reports of gynaecomastia^{5–7} (breast development) in human males indicate that given sufficient levels of oestrogen and progesterone, mammary glands will undergo hypertrophy and hyperplasia. Elevated circulating oestrogen levels may result from: liver malfunction, which can hinder its inactivation and clearance; from 5 α -reductase deficiency, in which failure to convert testosterone to dihydrotestosterone provides excess substrate for aromatization of androgen to oestrogen; or from dietary or topical exposure to phytoestrogens. Another possibility is that in male *D. spadiceus* mammary glands contain an aromatase system that converts circulating testosterone into oestrogen, in the same way that the hypothalamus of a neonatal male converts circulating testosterone locally to oestrogen during hypothalamic masculinization. If, in addition, there are high circulating levels of progesterone derived from the adrenal gland, as in some species of *Pteropus* (L. Martin, personal communication) and circulating levels of prolactin are adequate, an endocrine status conducive to lactation can be imagined.

Studies on circulating hormones in this

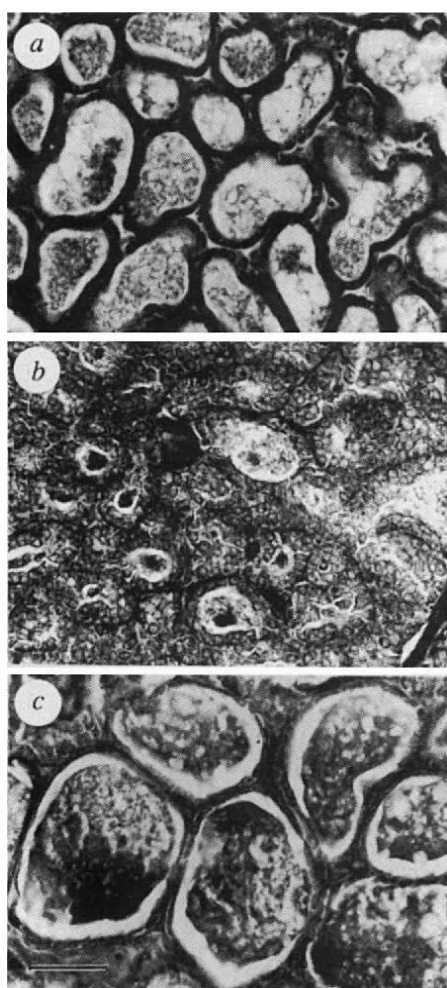


FIG. 1 Histological sections of mammary gland alveolar tissue in a lactating female *D. spadiceus* (a), a non-lactating female (b) and a lactating male (c). The glands were fixed by immersion in 10% buffered formalin, passed through a dehydrating series of ethanols followed by xylene, and embedded in Paraplast Plus. Sections were cut to a thickness of 10–15 μ m and stained with Delafield's hematoxylin and eosin. Bar, 50 μ m.

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bat will help clarify the physiological basis for male lactation. On theoretical grounds, functional male lactation would be most likely to evolve in monogamous species¹, in which males share in the care of the young and have high certainty of paternity. Studies of the social structure of *D. spadiceus* are required to determine whether they fit these criteria, and whether they actually provide young with milk.

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Antediluvian DNA research

SIR — Lindahl, in a Review article¹ and in Scientific Correspondence², has discussed the limitations of techniques to recover DNA from ancient and fossil samples, in this context citing my PCR amplification of part of a chloroplast gene from a 17-million-year-old fossil leaf³ as being "incompatible with the known properties of the chemical structure of DNA." His criticisms are based on controlled *in vitro* studies of rates of DNA depurination and subsequent chain breakage under physiological solvent conditions under high temperature^{4,5}. From these experimental trials, he extrapolated rates of depurination and suggested that they are inconsistent with long-term preservation of DNA. It is unfortunate that Lindahl either ignored or was unaware of various empirical reports that are consistently incompatible with his expected properties of DNA decay. Some of these reports were pointed out by G. Poinar, in a reply to Lindahl's Scientific Correspondence².

The most consistent finding from various studies on ancient DNA is that the rate of decay of DNA is not linear over time. Pääbo⁶ reported that, in DNA extractions from Mammalian soft tissue ranging in age from 4 to 13,000 years old, the extent of the size reduction of preserved DNA was independent of age, the preponderance of the damage occurring immediately post-mortem due to autolysis. Rapid desiccation appeared to improve preservation, although subsequent oxidative damage to the thymines occurred in all samples over time. The amount of ancient DNA in bones is not appreciably different in 200- and 9,000-year-old

samples⁷, and DNA preservation in ancient seeds is clearly not linear⁸. Within a shorter time frame, there is variability in DNA preservation which was not correlated with age⁹.

Of more direct relevance to the question of preservation of plant fossil DNA from the Clarkia deposits, Soltis *et al.*¹⁰ reported amplification of a 1,320-base pair fragment from a *Taxodium* Clarkia fossil leaf extraction. The analysis of the sequence clearly demonstrated that the sequence was not a contaminant sequence from an extant sample and showed a high degree of similarity to the expected congener.

Lindahl's reference to the work by Sidow *et al.*¹¹ must also be assessed in its full context. Sidow *et al.* failed to amplify chloroplast sequences from five leaf samples and one acorn, only two of which had DNA that was visible under ethidium bromide staining. They were successful in amplifying a mixed population of apparent bacterial DNA fragments. This success, however, did not correlate with the presence or absence of high molecular mass DNA, as all the samples, including those not having observable DNA and those derived from the adjacent shale without fossil leaf material, could successfully be amplified. The only conclusions that can be drawn from this work are that not all samples are readily amplifiable for chloroplast sequences under the conditions used, and that DNA from soil bacteria is also found in soil-derived samples.

In my view, the most distressing aspect of Lindahl's analysis is his failure to mention that genic DNA contains information that can be analysed by evolutionary systematics. The presence or absence of contaminating DNA is irrelevant to the question of the persistence of fossil DNA, as long as contaminants can be clearly identified. When there is contaminating DNA, analysis of the derived sequence readily identifies the data as false-positive. Even in the few cases in which the contaminating sequence cannot clearly be ascribed to exogenous sources, unexpected phylogenetic relations will indicate a false-positive. It is clear that presently used criteria for validation of ancient DNA are conservative both because of experimental design and because of the stochastic processes of nucleotide substitution, and thus will have a greater tendency towards rejecting true results than towards accepting false-positives¹².

Theory is important for generating testable predictions, and the validity of a theory is determined by how well it is supported by empirical results. The reverse of this process, establishing the validity of empirical results by determining how well they fit theoretical expectations, is at best arrogant, and at worst, regressive. It is clear from a growing body of empirical studies that the preservation

of DNA is not simply a function of time. Microenvironmental conditions within preserved tissues and organelles are likely to be vastly different from physiological conditions, making simple extrapolations from *in vitro* studies uninformative.

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Chimpanzee DNA profiles on trial

SIR — The capture and trading of great apes has been banned in 112 countries since a CITES meeting in Washington in 1973. Despite this agreement, about 1,000 chimpanzees are deported annually from Africa to Europe, the United States and Japan¹. Private owners (zoos, circuses) disguise this illegal trade by simulating births in captivity and by replacing adults with young animals. Measures to identify captive chimpanzees and control their numbers have so far had little effect. Genetic identity tests — the obvious solution — have never been used because of the unavailability of highly polymorphic markers and the inherent difficulties of obtaining blood samples (in most cases, blood is withdrawn under narcosis).

The use of highly polymorphic DNA markers has been proposed², in combination with the noninvasive procedures of genomic sampling^{3,4}. However, some of these markers (restriction-fragment length polymorphisms, multilocus and single-locus minisatellites) require large amounts of DNA, are labour-intensive and their use as identification profiles is controversial. Others (dinucleotide-repeat microsatellites⁵) are prone to errors in genotype diagnosis. If the standards of reliability and statistical parameters demanded for forensic analyses in humans⁶ were adopted for *Pan troglodytes*

dytes, they would probably not provide reliable results in parenthood tests and in individual profiling. Consequently, forensic evidence of chimpanzee deportation has so far remained elusive.

We report here a protocol for DNA profiling in common chimpanzees, based on DNA markers currently used for identification in humans, and the successful application of this technique to legal controversies involving animal identity. DNA samples from 58 *P. troglodytes* individuals were extracted from freshly plucked hair roots (the amount of genomic harvest largely varied from 10 to 160 ng per root; to ensure the feasibility of noninvasive sampling, no blood samples were made available) and used as templates for a series of polymerase chain reactions (PCR) primed by oligonucleotide pairs flanking human tandemly repeated polymorphisms. Six tetranucleotide-repeat microsatellites (VWF, 12p12–12pter (ref. 7); SE33, Chr 6 (ref. 8); FESFPS, 15q25qter (ref. 9); HUMTH01, 11p15.5 (ref. 10), MBP1, MBP2, 18q22–qter; ref. 11) and one AmpFLP (COL2A1, 12q14.3; ref. 12) were found to be polymorphic in *P. troglodytes*. The seven loci share remarkable analogies with humans and can be reliably typed by the same standard procedures (Fig. 1). Studies in sire–dam–offspring triads suggested a mendelian inheritance pattern and an

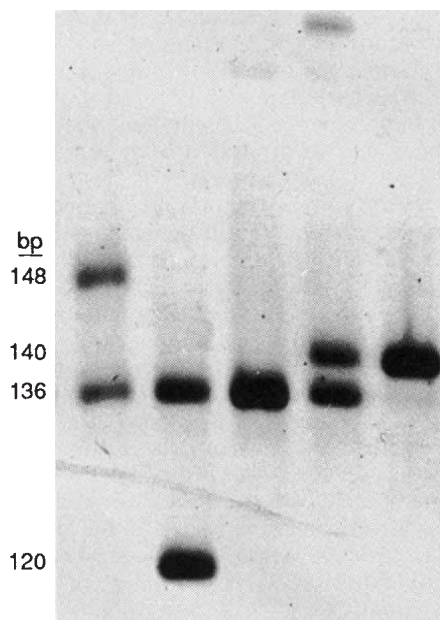


FIG. 1 Parental exclusion in a case of illegal chimpanzee trading. Aliquots of 20 ng genomic DNA, extracted from hair samples, were used as template in PCR reactions, using MBP1 primer pairs¹¹. Amplified products were run on a polyacrylamide gel and silver-stained. From left, sire, dam and three putative offspring (compatible, excluded at one parent, excluded at both) are shown. One-parent exclusion was rejected as proof of illegal trading. Court decisions are usually based on both parents' incompatibility.

MBP1/MBP2 (coamplified): 5' -GGACCTCGTGAATTACAATC -3'
5' -ATTTACCTACCTGTTTCATCC -3'
COL2A1: 5' -CCAGGTTAAGGTTGACAGCT -3'
5' -GTCATGAACTAGCTCTGGTG -3';
FESFPS: 5' -GGAAGATGGAGTGGCTGTTA -3'
5' -CTCCAGCCTGGCGAAAGAAT -3'
VWF: 5' -CCCTAGTGGATGATAAGAATAATC -3'
5' -GGACAGATGATAAATACATAGGATGGATGG -3'
SE33: 5' -AATCTGGGCGACAAGAGTGA -3'
5' -ACATCTCCCTACCGCTATA -3'
HUMTH01: 5' -GTGGGCTGAAAAGCTCCCGATTAT -3'
5' -ATTCAAAGGGTATCTGGGCTCTGG -3'

	Hetero zygosity	Size (bp)	1	2	3	Alleles: 4	5	6	7	8
COL2A1	0.73	200-480	0.061	0.348	0.030	0.015	0.242	0.015	0.061	0.152
VWF	0.66	110-150	0.184	0.158	0.119	0.25	0.066	0.171	0.053	0.013
MBP2	0.65	210-230	0.097	0.081	0.177	0.323	0.274	0.032	0.016	
MBP1	0.52	120-150	0.120	0.040	0.200	0.120	0.300	0.200	0.020	
FESFPS	0.50	230-250	0.028	0.361	0.014	0.597				
SE33	0.36	220-250	0.180	0.700	0.120					
HUMTH01	0.29	160-180	0.107	0.821	0.018					

FIG. 2 Frequency of alleles at seven polymorphic loci of *P. troglodytes* genome (45 non-inbred individuals). Alleles are arbitrarily numbered according to decreasing size.

apparent equilibrium of gametic phase. Although tested on individuals derived from probably more than one subgroup and from different troops (all individuals were detained in Italy; 48 individuals were wild-caught and supposedly came from Sierra Leone, Congo and Nigeria), genotype distribution showed negligible deviation from the Hardy–Weinberg equilibrium. The combined use of the seven polymorphisms enabled us to specify a quasi-unique identity code (Fig. 2), in which the most common haplotype would theoretically occur every 2.8×10^{-5} individuals. The *a priori* probability of excluding false parenthood¹³ was also significant (~99%), and bayesian estimates of motherhood in dam–offspring pairs were possible. The whole pool of markers was typed from 150-ng nuclear DNA, a quantity equal to the extract of a few hair roots.

On this basis, we decided to introduce identification profiles and parenthood tests in legal cases. We have provided evidence leading to a conviction for a false veterinary certification of a birth in captivity (Verona, 1992), and in seven further cases, illegal deportation was reported by CITES officials after DNA analysis on plucked hairs. The Italian CITES authority has established a repertory of DNA profiles from *P. troglodytes* individuals detained in Italy. These data will become a permanent record of captive chimpanzees and will enable comparisons with matching profiles. We hope that extensive control of animal DNA profiles will reduce, and eventually eradicate, the prac-

tice of illegally introducing wild specimens into the country.

The seven polymorphisms may have further application in studies on the demography, ecology and perhaps taxonomy of wild chimpanzees. Further details (formal and population genetics, map position, sequence, tandem-repeat hierarchy) will be published elsewhere.

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Less mercury?

SIR — Camargo¹ dismisses Nriagu's² assertion that mercury emissions from precious-metal refining in the early New World may have an impact on the present-day global mercury cycle. Unfortunately, Camargo's argument is undermined by the use of outdated, poor-quality data collected before people became aware of the need for ultra-clean sample-handling protocols³⁻⁵.

Camargo, for example, cites a 15-year-old EPA report⁶, founded on much earlier data, for his assertion that the total natural-source atmospheric Hg emission budget is $25-50 \times 10^9 \text{ g yr}^{-1}$. But it is now generally accepted that the total global atmospheric emission budget is about $6-7.5 \times 10^9 \text{ g yr}^{-1}$, with approximately 30-75% of that as natural emissions, and perhaps as much as 20-30% due to fossil fuel combustion⁷⁻¹¹. Using these values results in more refined estimates of the atmospheric residence time for Hg of 0.5-2 yr (refs. 7, 8, 10).

Camargo also overestimates the potential pool of Hg contained in the oceans by two orders of magnitude, choosing very old and discredited values as representative of the world oceans^{12,13}. Using ultra-clean techniques many researchers have now confirmed that ocean water, even near the shore, contains approximately $0.5-1 \text{ ng l}^{-1} \text{ Hg}$ (refs 4, 7, 14), resulting in an estimated 10^{12} g Hg residing there at any one time. From this, we can calculate a mean oceanic residence time for Hg of approximately 350 yr, which is comparable with other particle-reactive metals in the sea¹⁵. This implies that much of the Hg emitted over the past 500 years still resides in the ocean, where it may be available for methylation, biological uptake, revolatilization, and recycling to the atmosphere^{7,16}.

Thus, modern and past anthropogenic emissions contribute to an increased global 'background' Hg concentration.

The degree to which the early emissions discussed by Nriagu affect the current budget is difficult to judge because of the incomplete summation of all Hg emissions from that time to the present, and our lack of knowledge as to the degree which Hg stored in deep ocean water is biogeochemically active and available for eventual revolatilization to the atmosphere. For example, it has recently been estimated that $6 \times 10^{10} \text{ g Hg}$ entered the environment in the nineteenth century via the recovery of gold and silver during the North American gold rush^{17,18}. But without a quantitative estimate for the summation of all releases during this interval it is impossible to compare its impact relative to the earlier and later South American gold rushes.

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Can diamonds be dead bacteria?

SIR — The reports by Lilley *et al.*¹ of isotopically very light carbon in methane from an unsedimented mid-ocean ridge system, and by Schrauder and Navon² of the discovery of solid CO₂ in diamond, reinvigorate an interesting question. Although Lilley *et al.* conclude that sediments from turbidites were a more likely source of methane and ammonia, their report raises the question of the long-term future of organic vent communities on unsedimented ridges, destined for eventual subduction. Are eclogitic diamonds fossils? Are they derived from metamorphosed clumps of carbon that originated as bacteria from ancient mid-ocean ridge hydrothermal systems?

The possible organic origin of some diamonds has been pointed out by Kirkley and Gurney³. To date, much of the discussion has centred around the possibility that organic material from sediments may have supplied the carbon. However, Deines *et al.*⁴, studying websteritic-type diamonds, discounted an organic-sediment origin for the isotopically light carbon on the grounds that a subduction hypothesis would demand an extreme ophiolite differentiate to explain the iron and chromium contents. Such differentiates are unlikely in association with sediments, but are expected near ridge hydrothermal communities.

The case for an organic origin rests

essentially on the isotope composition of the carbon in the diamonds occurring in eclogites, which commonly ranges from $\delta^{13}\text{C}$ of 0‰ to as light as -25‰, and in rare examples yet lighter, to -35‰. The main source of carbon as light as this is organic. Bacteria show a wide range of carbon isotope compositions. Isotope fractionation in the Rubisco pathway is about -29‰, which with the chemical fractionation between dissolved CO₂ and carbonate (about 11‰) allows a maximum biological fractionation of about -40‰. Perhaps more relevant to the proposed hydrothermal setting is that archaea (such as methanogens) can range further, to extremely light $\delta^{13}\text{C}$. Further, the sulphur isotope ratios of many sulphide inclusions in eclogitic diamonds suggests a biogenic origin⁵ and the high manganese content of some diamondiferous eclogites and eclogitic inclusions in diamonds⁶ is not inconsistent with a hydrothermal source.

Late Archaean and Proterozoic oceanic crust would have contained a thick mafic upper layer, with vigorous hydrothermal circulation. Abundant vent communities may have existed, becoming incorporated into an altered carbonate-rich lava pile and eventually subducted. It is then logical to ask what happened to the bacterial communities that are widely assumed to have been present on Archaean and Proterozoic mid-ocean ridge hydrothermal systems? A possible answer is that some diamonds derived their carbon from bacterial communities, either directly from reduced organic carbon via graphite, or indirectly via a locally generated gas phase that incorporated both very light organic-derived carbon and also CO₂ from carbonate. The original organic carbon may have been in the range -30 to -50‰. Metamorphism tends to shift organic carbon to heavier isotope ratios. The present isotope content of the diamonds would range from near-organic ratios (-35‰) to more common heavier compositions (0 to -25‰) that reflect varying degrees of metamorphism and exchange with country rock carbon from carbonate. There are probably many origins of the carbon in the various types of diamonds, but it is possible that a few diamonds are indeed our ancestral relics.

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Our changing environment

Ralph J. Cicerone

Atmospheric Change: An Earth System Perspective. By T. E. Graedel and P. J. Crutzen. W. H. Freeman: 1993. Pp. 446. £35.95, \$42.95 (hbk); £16.95, \$32.95 (pbk).

'EARTH system science' is a term being adopted by teachers and researchers who are concerned with more than just oceanography, meteorology, hydrology and surface geology, and who are attempting to understand environmental changes due to natural and human-induced disturbances. The agenda of Earth system science includes estimating how global and regional climates and biota will change as a result of the infrared radiative 'forcing' from increased concentrations of greenhouse gases in the atmosphere; the extent of present and future ozone-layer losses and the effects of increased ultraviolet light dosages on humans and other organisms; how air, oceans and soils are changing as humans accelerate their manipulations of the cycles of several nutrient elements such as nitrogen, sulphur and phosphorus; and how volcanoes affect atmospheric chemistry and the Earth's climate. Despite the increasing importance of such questions, a scientific framework for their investigation has been difficult to construct. Although much progress has been made through disciplines such as physical oceanography or atmospheric chemistry, many feedbacks are obvious in the system and some broader framework is needed. Probably the only integrated concept of the whole system's behaviour is Gaia from James Lovelock; Gaia has demonstrated the importance of the biota but it is not serving as the general prism through which all physical, chemical and biological phenomena and today's questions can be viewed by Earth scientists.

Graedel and Crutzen's book, subtitled "An Earth System Perspective" does an amazing job of introducing the reader to the large-scale issues of today and the future, and to the chemistry, physics and (less so) biology that will determine how the system responds. The book's scope and its range of examples impress on the reader the scientific challenge ahead and also the intellectual breadth of the authors. In discussion of aquatic phenomena, for example, an enormous range of concentrations, oxidation states and anions and cations is observed in waters from rain, clouds, fog, dew, groundwater, oceans and brines; both thermodynamic and kinetic analyses are used to make sense of these arrays. Similarly, palaeorecords such as those of gases trapped in ice cores are introduced to estimate the limits and the timescales of past Earth changes. Interesting measurement records are pre-

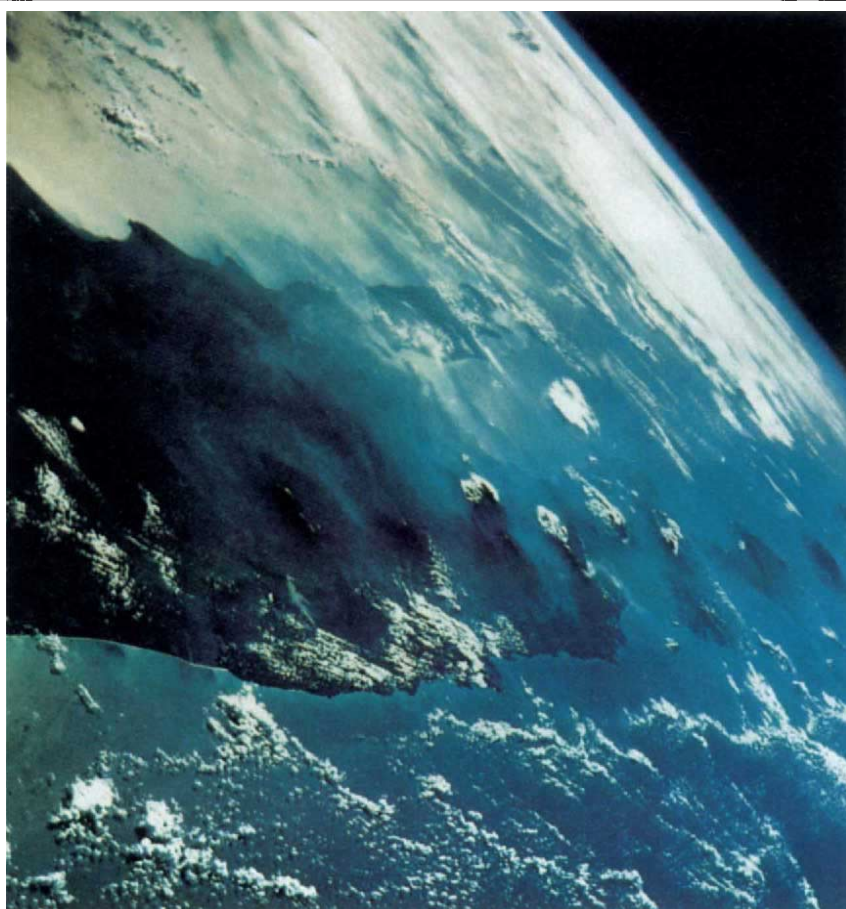
sented from around the world; Earth system science knows no boundaries (and the authors seem aware of the best data regardless of their source). Spatial scales in physical phenomena from global to subatomic are involved in atmospheric and environmental change, as are many temporal scales; the authors present modelling approaches to take these into account as they synthesize, explain and attempt to predict future changes. The book is accurate and careful throughout: for example, the dot notation is used to denote free radicals, a practice that is neglected in most other books and journal articles. In almost all cases, data are also up to date.

Who will use this book? It would be best

for a course (with the same title as the book) for motivated, advanced undergraduates, intended to provide a foundation and broad context for further evaluations of human impacts and of the limits of stability of the natural system. Such a course would be at least 15 weeks long. Because of its emphasis on issues, I think that *Atmospheric Change* is more motivating than *Global Biogeochemical Cycles* (eds S. S. Butcher *et al.*, Academic Press, 1992). Alternatively, parts of the book could serve as text material for a briefer course on atmospheric and environmental chemistry.

The authors express concern about human population growth and poor environmental stewardship. Given that scientific data and understanding are necessary to deal with these issues, and that excellent books contribute to this knowledge, the authors have done their share toward solving our problems. □

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This view of a remote volcanic island chain in Eastern Java was taken from the Space Shuttle in 1985. Observations from space are often the best way of learning which volcanic gases and aerosols are injected into the atmosphere, the thermal structure and volume of active lava flows, and the deformation history of volcanic cones and craters. The picture is taken from *Atlas of Satellite Observations Related to Global Change* edited by R. J. Gurney, J. L. Foster and C. L. Parkinson, a richly illustrated account for a general scientific audience. Cambridge University Press, £35, \$49.95.

Testable claims

Lee Loevinger

Being an Effective Expert Witness: The Technologist in the Courtroom. By Derek A. Smith. Thames Publishing, 14 Barlby Road, London W10 6AR, UK. 1993. Pp. 297. £34.50.

DEREK Smith is a chemist, an experienced witness and a natural raconteur, who reports years of experience with literary flair. His account is particularly timely: the subject of scientific testimony has become of widespread interest in the United States following the decision in June 1993 by the Supreme Court in *Daubert v. Merrell Dow Pharmaceuticals* promulgating standards for admissible scientific evidence. *Shepard's Expert and Scientific Evidence Quarterly* has begun publication, and the American Association for the Advancement of Science and the Science and Technology Section of the American Bar Association have held a conference with the Federal Judicial Center to start a long-term experiment using Federal Rule of Evidence 706 to encourage court appointment of neutral scientists in appropriate cases. Articles and cases citing *Daubert* are beginning to proliferate.

Most of what is being written is by

lawyers (including some judges) who explain the law to scientists or to each other. Smith is a scientist who has undertaken to explain science to lawyers and legal procedure to scientists with specific advice as to how to serve as an expert witness. His style is informal, idiomatic and lightened by frequent quotations from sources as diverse as the Bible, Shakespeare, Lewis Carroll, and Gilbert and Sullivan, some in footnotes, which are often as amusing as the text. More notably, his observations are illustrated by anecdotes of actual cases, mainly ones in which he has participated.

Smith writes in an English (as distinct from American) vernacular, referring to lawyers as solicitors, barristers and counsel (the barrister pleading a case), but the book is understandable and useful to an American interested in the subject, and there is a short glossary for those confused by the terminology. Anyway, the basics of English and American litigation are the same. The federal courts in the United States correspond to the High Court in England, the larger and more important cases tend to be filed in such courts, and about the same number of cases are filed in these courts annually in each country. More of the cases filed go to trial in the United States than in England, but the number is less than 5 per cent in both countries.

The big difference between the legal

systems lies in the existence of the 50 state court systems in the United States. In England about 10 times as many cases are filed each year in the county, or lower, courts as in the High Court. In the United States more than a hundred times as many cases are filed each year in the state courts as in the federal courts.

The relationship of scientific and technical experts to lawyers and courts is much the same regardless of the court in which a case is filed. Smith relates experience in English, US and continental courts, and notes the variations. The major differences he has observed between English and American lawsuits is that in the latter there is more pre-trial discovery, including depositions; higher attorney fees; more incivility between adversaries, including more hectoring of witnesses on cross-examination; and there have been lower standards for scientific experts permitting more testimony by bogus scientists. (A footnote adds that in the United States more corporate executives have given up smoking!) Although the forms of legal procedure differ among the countries, the viewpoints and roles of scientists appear generally uniform in the Western world.

American lawyers may get some feel for the atmosphere and pace of English litigation from the descriptions in this book, but it is definitely not a lawyer's guide to English practice. Many cases are referred to by title but no legal citations are given. Although the principles expounded are valid for all varieties of expert testimony, the focus is on patent cases involving issues of chemical compounds, processes or materials. Nevertheless, the book is primarily what its title suggests — a guide to expert witnesses in technological cases. This function it fulfils very thoroughly. Both experienced and merely expectant expert witnesses will profit from reading it, as it systematically covers all aspects of that complex and important role. Lawyers and business executives who have occasion to deal with expert witnesses, or with scientific consultants, will gain valuable insight into the attitudes of scientists.

Every trial lawyer eventually formulates his own instructions to experts and other witnesses, but it is most unlikely that any has prepared such an extensive dissertation. Some may hesitate to require prospective witnesses to read such a lengthy exposition because that will increase the expert's fees. However, the book can be recommended without reservation — as a trial lawyer I found nothing in it with which to disagree.

Although there is little philosophizing, Smith understands and discusses the difference between courtroom testimony and scientific publication. The last chapter considers the future of the expert witness, noting that the resolution of disputes



CONVOLUTIONS of a smooth-lipped shell — *Phyllonotus regius* (Muricidae; $\times 0.8$) from Playa Brava, Costa Rica. In *A Natural History of Shells*, from which this photograph is taken, Geerat J. Vemeij provides an elegantly written and beautifully illustrated account of shell construction, function and evolution, while showing how these molluscan houses give us insights into ecology and the history of life. A book that will be treasured by scientists and lay readers alike. Princeton University Press, \$29.95, £22.50.

through the use of experts is firmly rooted in our culture. (Legal historians say the practice dates from the fourteenth century.) The broadening scope of subjects in which expertise is now needed is briefly surveyed; and Smith ends by quoting approvingly a leading article in *Nature* (362, 481; 1993) recommending that scientific evidence should be admitted in court only if the claim is testable, it has been

tested, and the methodology is sound. This is close to the conclusion reached by the US Supreme Court. The book therefore describes the kind of scientific testimony the courts now require. □

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On the evolutionary treadmill

Magnus Enquist and Risa Rosenberg

The Red Queen: Sex and the Evolution of Human Nature. By Matt Ridley. Viking: 1993. Pp. 405. £17.99.

In Lewis Carroll's *Through the Looking Glass*, the Red Queen perpetually runs yet forever remains in place. She has become a metaphor for a problem in

This problem is the subject of Matt Ridley's book. The reader is treated to an intellectual journey focusing on sex and sexual behaviour in an attempt to define human nature. The first third of the book perceptively concentrates on basic descriptions and why sex occurs as seen from the different viewpoints of molecular biologists, geneticists and ecologists.

Later chapters question why there are different sexes, and how animals choose and acquire sexual partners. It is not until midway through the book that human nature is addressed. The last part of the book takes us through discussions of how the brains of males and females differ, the meaning of beauty, male and female reproductive strategies, intelligence, and, of course, how all these issues relate to matters of sex.

There is a wealth of information here, and it is an excellent source for researchers because of the descriptions of studies and its extensive reference section, as well as being an interesting book for a scientifically literate public. The book is entertaining, with such descriptive phrases as "amorphous blobs of insectness" (scale insects), and information from *Romeo and Juliet*, *Julius Caesar*, *James Bond*, *Cain and Abel*, and a number of meetings with Mar-

tian scientists. The quality of writing only rarely falters.

A book that discusses human nature cannot avoid being speculative, given the present scarcity of knowledge about this topic. The author makes no secret of his adaptationist leanings, and unfortunately the adaptationist programme is sometimes pushed a bit too far, with arguments raising more questions than they answer. For instance, the provocative discussions

of the ideal (thin-waisted) female form, true Nordic blondes and homosexuality are treated rather narrowly and not always convincingly.

We are most troubled, however, by the seminal role Ridley has given the Red Queen in the evolution of humans. He claims that human nature is primarily a product of sexual rather than natural selection, and that human nature is mainly a result of competition for mates in an evolutionary race that will both never end and never lead anywhere. This conclusion is difficult to accept. The fact is that in humans the Red Queen seems to have outrun her surroundings; humans have become one of the most successful species of all time, in terms of numbers and distribution. Such success cannot be explained by sexual games. Individuals capable of making more efficient use of their environment and those able to cope with new environments must have been favoured by selection.

We look forward to a time when we really know the answers to some of the questions raised by the author and the extent to which adaptive explanations are valid. Ridley's book and others in this genre go far in stimulating interest in evolutionary biology which is crucial to anyone seriously interested in human nature. □

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Cultured minds

Chris Stringer

The Runaway Brain: The Evolution of Human Uniqueness. By Christopher Wills. BasicBooks: 1993. Pp. 358. \$25.00. To be published in the United Kingdom by HarperCollins on 24 March.

THIS is an attempt to explain what factors lie behind human evolution. Wills argues that "what is unique [about our evolution] is not the process, but the result. That is why the subtitle of this book is *The Evolution of Human Uniqueness*, not the *Uniqueness of Human Evolution*." He follows an initially rather conventional view that a culture-brain feedback loop was established early in human evolution, and that this was fuelled by the stimulating effect of the African environment. In his view, through selection for the increased power of the human brain to process and synthesize vast amounts of stored and new data, and for longer and deeper spells of concentration, the feedback loop gradually accelerated to a runaway pace.

The book covers a lot of ground, much of it a familiar retelling of the historical



Going nowhere fast — Alice and the Red Queen.

evolutionary biology. The success of an organism is partly influenced by the strategies used by others around it (frequency-dependent selection). Such problems may lead to evolutionary equilibria and, in other cases, there will be an evolutionary race with continuous changes in counteracting strategies. Sometimes, as the Red Queen found, such races will seem meaningless as there seems to be little progress.

development of palaeoanthropology (the Neander Valley, Dubois' "*Pithecanthropus*", Piltdown, Dart's *Australopithecus* and so on). But there are also excursions into less standard territory such as Krech and Rosenzweig's psychological experiments on rats, the bacterial ancestry of mitochondria, Muller's ideas on eugenics, Dobzhansky's research on fruitfly chromosomes and Kimura's neutral theory. While these certainly make fascinating reading for the uninitiated, it is not always clear how they relate to the main theme of the book.

Wills supports the concept of a multi-regional evolution of modern humans and treats Coon's generally discredited notions of separate racial origins from the *Homo erectus* stage with some sympathy. He believes that most of our evolution had occurred before *Homo erectus*, and run-away cultural and brain evolution would already have been set on its course in the different *H. erectus* populations, programming them to become *H. sapiens* independently (following Coon's model) or semi-independently (with gene flow). I think he considerably weakens his case by restricting the term 'human' to 'modern' *H. sapiens*, instead of using it in the wider sense as synonymous with members of the genus *Homo*; for Wills, even the Neanderthals are not fully 'human', creating a sharp division which he otherwise seems to imply does not really exist. Part of his support for multiregional evolution stems from his reanalysis of mitochondrial DNA data, concentrating on the rarer transversions which are otherwise usually swamped by transitions. His resulting recalibration of the age of the hypothetical 'mitochondrial Eve' to 600,000–1,000,000 years is about four times the better known (and now hotly disputed) estimates of workers such as Cann, Stoneking and the

late Allan Wilson.

Wills's discussion of the complexities of genes and brains is clear, although the symbolic power of language to change dramatically the way humans thought could have been given more emphasis. But Wills is sometimes less successful in dealing with the fossil evidence. For example, it is arguable whether the australopithecines "resemble us much more closely than they do apes", and there is no strong evidence that Africa lagged behind the rest of the world during the later parts of the Old Stone Age. (Some data suggest quite the opposite.) His assessments of dating are sometimes also faulty: the bones of the "First Family" are not younger than Lucy's, nor is the Laetoli site very close to her in age; and electron-spin-resonance dates from Kebara are not double the thermoluminescence dates (in fact they match well).

Overall, this book provides a distinctive perspective on human evolution, although ultimately it is not very different in its conclusions from the familiar views of palaeoanthropologists such as Milford Wolpoff and Phillip Tobias. Wills certainly puts too much store by his recalibration of the age of 'Eve' in reaffirming the reality of Coon's and Wolpoff's models; and, having been critical of those who put too much reliance on previous work on mitochondrial DNA, it is a pity that he does not take a wider view of genetic reconstructions of recent human evolution from nuclear DNA as well. Nevertheless, the volume is an interesting, if at times idiosyncratic, addition to the continuing flow of books on human evolution. □

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New in paperback

Complexity: The Emerging Science at the Edge of Order and Chaos by M. Mitchell Waldrop. Penguin, £6.99. See *Nature* **361**, 507 (1993).

Complexity: Life at the Edge of Chaos by Roger Lewin. Macmillan Publishing Company, New York, \$10. See *Nature* **361**, 507 (1993).

Creation Revisited: The Origin of Space, Time and the Universe by Peter Atkins. A defence of extreme reductionism and militant rationalism, and the subject of much debate when first published in 1981. See *Nature* **294**, 595 (1981). Penguin, £6.99.

The Origins of Chemistry by Robert P. Multhauf, first published in 1966 and described in *Nature* as "a professional's book for professionals, but . . . it will bring great pleasure to anyone who can claim to be scientifically educated". Gordon & Breach, \$48, £31.

Bright Air, Brilliant Fire: On the Matter of the Mind by Gerald Edelman. A Nobel prizewinner's attempt to present his controversial theory to a general audience. Reviewed in *Nature* **360**, 426 (1992). Penguin, £7.99.

Uncertainty: The Life and Science of Werner Heisenberg by David C. Cassidy. One of the best scientific biographies published in recent years. Reviewed in *Nature* **354**, 365 (1991). W. H. Freeman, \$19.95, £15.95.

On Methuselah's Trail: Living Fossils and the Great Extinctions by P. D. Ward. "As unputdownable as any airport-lounge thriller" (*Nature* **355**, 600; 1992). W. H. Freeman, \$12.85, £11.95.

Taming the Atom: The Emergence of the Visible Microworld by Hans Christian von Baeyer. A lucid history of atomism from the Greeks to the present, favourably reviewed in *Nature* **361**, 215 (1993). Penguin, £7.99.

Elements on disc

Tony Cox

Interactive Periodic Table on CD-ROM. ATTICA Cybernetics, Oxford, UK, in association with the Department of Chemistry, University of Southampton. £116.33. US distributor, Compton's New Media Inc. (619 929 2555).

THE chemical elements are fascinating in their diversity. Even within the organizing framework of the Periodic Table, their properties can show startling differences between one element and the next. All good books and courses on inorganic chemistry try to bring out this diversity, and an interactive teaching aid on CD-ROM would seem to be an ideal medium to help students to explore it on their own. The contents of this package are themselves bewilderingly diverse. They include Tom Lehrer's 'The Elements' song, video excerpts and poems, a brief tutorial guide to the Periodic Table and the discovery of the elements, and a huge amount of hard data, which can be displayed in all sorts of ways. My first reaction was that the product does not hang together very well. A more charitable interpretation is that the authors have included material that will be useful to students at different levels.

The more elementary parts, suitable for school chemistry or the first year at university, work fairly well. Whether one really needs this kind of audiovisual material on a CD-ROM, rather than say a videotape, is less clear. The CD format does make it much easier to work interactively, scanning through elements in a chosen order, rather than one imposed by the producer. But the quality and scope of the visual material suffers. I have greater reservations about the data intended for more advanced students. Many kinds of display are possible; for example, one can plot the ionic radius of elements against their abundance in the Earth's crust if one really wants to, although the visual quality of the graphs is disappointing. Also, the contents of the database are somewhat erratic, giving for instance an ionic radius for Ni^{5+} but not for Br^- .

Personally I prefer a good book, but I am aware of a generation gap here, and a computer-based medium like this undoubtedly has a growing role to play in the teaching of chemistry. As an introduction to the Periodic Table and to some descriptive aspects of the elements, the package is attractive. As a database and means of displaying more quantitative relationships, it probably needs more work before it can be really useful. □

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The role of metamorphic decarbonation reactions in returning strontium to the silicate sediment mass

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The strontium-isotope evolution of the Earth's silicate sediments indicates that they must interact with a strontium reservoir whose $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is relatively low. The Sr-isotope budget for these sediments can be balanced if the Sr lost to solution by weathering is replaced by Sr from the oceans (through diagenesis) and from carbonate sediments (through metamorphic decarbonation reactions). Because of the latter connection, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of silicate sediments can constrain the contribution of metamorphism to the Earth-atmosphere CO_2 flux.

THE $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratio is one of the key tracers for a range of crust-mantle-hydrosphere geochemical interactions, because rubidium (^{87}Rb decays to ^{87}Sr) is significantly fractionated from strontium in different geochemical reservoirs. A striking example of this is the high Rb/Sr ratio in fine-grained silicate clastic sediments, which reflects the affinity of Rb (with K) for clay minerals, and the loss of Sr (with Ca) to solution during weathering (Fig. 1). Because silicate sediments have high Rb/Sr ratios, they develop high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios over time from ^{87}Rb decay (Fig. 2a) and, because new silicate sediment is derived predominantly by erosional recycling of existing sediment, the initial $^{87}\text{Sr}/^{86}\text{Sr}$ of newly deposited silicate sediments might be expected to increase with time. But, as first pointed out by Goldstein¹, the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of silicate sediments have remained relatively low and constant over the Phanerozoic eon (Fig. 2b). Goldstein speculated that inputs of low $^{87}\text{Sr}/^{86}\text{Sr}$ material from new crust might be sufficient to buffer sedimentary initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios although he did not consider the question in detail. I suggest instead that the loss of relatively radiogenic Sr by weathering must be balanced by a return flux from a less radiogenic source, and I identify this source as the ocean/carbonate reservoir. Some of this strontium is transported from carbonate to silicate minerals by diagenesis, and the rest by silicate-producing carbonate breakdown reactions in metamorphic belts. The strontium-isotope budget of silicate sediments has significance beyond the chemical fluxes involved in sedimentary recycling. The Sr isotope composition of sea water preserves a finely resolved record which reflects variations in the supply of radiogenic Sr from the continents and unradiogenic Sr from oceanic crust^{2,3}. Interpreting this record requires an understanding of the processes that moderate crustal $^{87}\text{Sr}/^{86}\text{Sr}$ ratios as well as those that alter erosion and weathering rates.

I also argue that the Sr isotope record can be used to constrain the fluxes of other chemically related species. Long-term atmospheric CO_2 concentrations are moderated by the balance between the adsorption of atmospheric CO_2 by weathering, its deposition as calcium carbonate, and the return of this CO_2 to

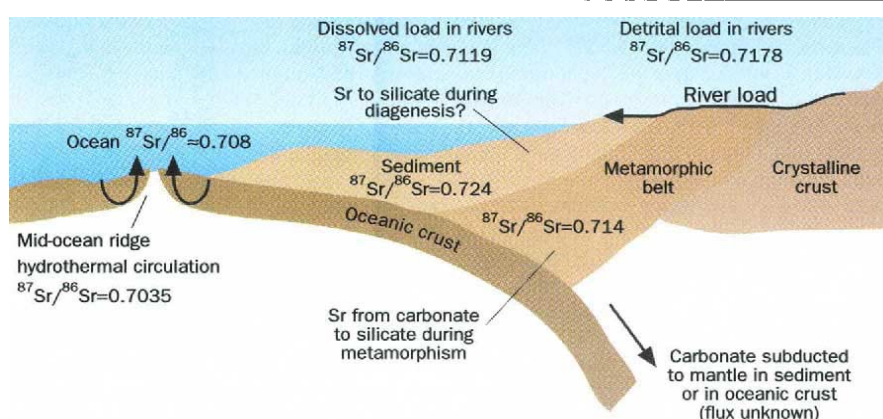
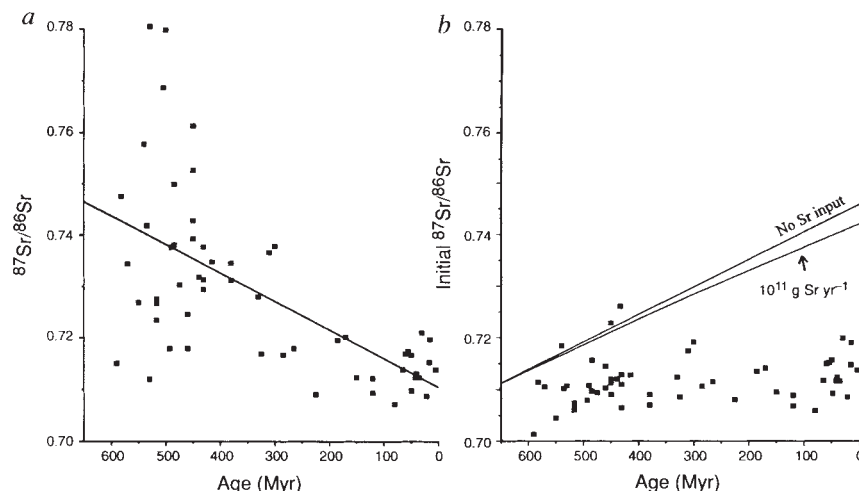


FIG. 1 Schematic illustration of the Sr cycle. The river load consists of a detrital load (derived from sediment, rapidly recycled metamorphics and a small component from new crust) and a dissolved load (derived from silicate and carbonate). About half the Sr in eroded silicate material, together with Sr from carbonate, goes into solution complexed with HCO_3^- ions to be deposited as carbonate in the oceans. Sr may be transferred back to silicate minerals by diagenesis, metamorphism, or as a result of carbonate in altered oceanic crust or sediment being subducted into the mantle. The Sr isotope composition of the oceans is maintained at $\sim 0.708 \pm 0.001$ as a result of the balance between dissolved Sr from the crust, and Sr input from hydrothermal circulation at mid-ocean ridges.

the atmosphere by decarbonation reactions during diagenesis, metamorphism or magmatism⁴. Much modelling of both long-term and short-term CO_2 -related climate fluctuations is concerned with interactions between various geological controls on weathering rates (such as sea level) and on orogenic uplift rates. Attempts to incorporate controls on the return flux of CO_2 to the atmosphere have generally been simplistic, because the mechanism is unknown. For example, Berner *et al.*⁴ presumed that all of the CO_2 returned to the atmosphere came from the mantle, through mid-ocean ridges, and was therefore proportional to sea-floor spreading rates. Sr is chemically similar to Ca, and I suggest here that Sr-isotope compositions have the potential to track interactions between silicate and carbonate sediments that may play an important role in controlling atmospheric CO_2 concentrations. I argue that the Sr isotope evolution of the Earth's silicate sediment mass can be used to monitor the magnitude and location of the decarbonation processes, and conclude that decarbonation reactions in metamorphic belts must contribute a significant proportion of the CO_2 flux from

FIG. 2 Sr isotope systematics of silicate sediments after Goldstein¹ but with expanded data set. $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of Phanerozoic silicate sediments plotted against stratigraphic age³⁹. Points represent averages from outcrops where multiple samples were taken. Data from refs 10–14, 40–57. Line is linear regression excluding two high $^{87}\text{Sr}/^{86}\text{Sr}$ points (one of which plots above diagram) and corresponds to an Rb/Sr ratio of 1.3 ± 0.4 (2σ). b, Initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of Phanerozoic sediments from (a) calculated from Rb/Sr ratios and stratigraphic ages. Two points below $^{87}\text{Sr}/^{86}\text{Sr} = 0.7$ not shown. Curves show $^{87}\text{Sr}/^{86}\text{Sr}$ evolution for silicate-sediment mass (4.5×10^{20} g Sr, Rb/Sr = 1.3) from initial $^{87}\text{Sr}/^{86}\text{Sr} = 0.711$ at 650 Myr with no Sr input, and with an additional input of 10^{11} g Sr yr^{-1} ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7035$) from new crust, to illustrate the Sr isotope evolution of the silicate sediment mass in the absence of other exchange processes.



the solid Earth to the atmosphere.

Sr isotope evolution of silicate sediments

The sediment mass contains 83% detrital silicate sediment, predominantly shale, and 17% chemical sediment, predominantly carbonate³. The strontium-isotope composition of detrital silicate material is little affected by the processes of weathering, erosion and transport in rivers and oceans⁶, and the Rb–Sr isotopic systems of the silicate and carbonate fractions of the sediment mass evolve independently. The volume–age distribution of the global sediment mass implies rapid recycling with a half-life of ~160 Myr (ref. 7); the recycling is largely cannibalistic, with much new sediment derived from erosion of pre-existing sediment⁸. Because silicate sediments have an elevated Rb/Sr ratio, they might be expected to evolve rapidly to high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios as are observed in older silicate sediments (Fig. 2a). But the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of silicate sediments (estimated from their present-day $^{87}\text{Sr}/^{86}\text{Sr}$ ratios and corrected for ^{87}Rb decay from their $^{87}\text{Sr}/^{86}\text{Sr}$ ratios and stratigraphic ages) are low and have remained constant over the Phanerozoic eon¹ (Fig. 2b). Regression of the data in Fig. 2a coupled with the mass–age distribution from Veizer and Jansen⁷ gives a mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio for the silicate sediment mass of 0.720 to 0.727, and a Rb/Sr ratio between 0.9 and 1.7. The mean initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the silicate sediments during the Phanerozoic eon from the data in Fig. 2b (0.7112 ± 0.0024 , $2 \times$ standard error, Fig. 3) and the mean $^{87}\text{Sr}/^{86}\text{Sr}$ of silicate detrital material being deposited today (0.7178 ± 0.0014 , Fig. 3) are both less than the estimate of the mean $^{87}\text{Sr}/^{86}\text{Sr}$ of the whole silicate sediment mass. The mean initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of present-day silicate detrital material is equivalent to the mean initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of silicate sediments being deposited now; this value may, however, differ from the average initial $^{87}\text{Sr}/^{86}\text{Sr}$ of Phanerozoic silicate sediments due to a variety of post-depositional processes or because of secular evolution of detrital silicate $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. Figure 2b indicates that the contribution from new crust with a mantle-like $^{87}\text{Sr}/^{86}\text{Sr}$ ratio cannot explain the Sr isotope evolution of the silicate sediment mass. The fraction of sediment derived from new crust is best constrained by the Nd isotope evolution of clastic sediments through time. All the studies indicate progressively smaller rates of new crustal additions to the sediment mass through time; Miller *et al.*⁹ estimate that only 6% of the sediment mass is derived from juvenile additions to the continental crust in the last 500 Myr. At this rate new crust is added to the sediment mass at 2.5×10^{14} g yr^{-1} , and if this contains ~400 p.p.m. Sr (ref. 5), only $\sim 1 \times 10^{11}$ g Sr yr^{-1} , $^{87}\text{Sr}/^{86}\text{Sr} = 0.7035$, are added. Even if this estimate were low by a factor of two, it is insufficient to cause significant modification of the Sr isotope composition,

and would not replace the Sr lost by weathering during erosive recycling of the silicate sediment mass.

Before addressing the problem of fluxes of Sr into the silicate-sediment mass, the difference between the estimated mean initial ratio of Phanerozoic silicate sediments (0.7112) and present-day mean silicate detrital material (0.7178) should be considered. Some of the difference results from the Sr in calcium carbonate in clastic sedimentary rocks. Leaching experiments demonstrate that most silicate clastic sediments contain a significant fraction of their strontium in carbonate which is not in isotopic equilibrium with the silicate minerals^{10–14}. The average shale contains ~6.6 wt% carbonate¹⁵ which, added to silicate detritus with $^{87}\text{Sr}/^{86}\text{Sr} = 0.7178$ would give bulk rock $^{87}\text{Sr}/^{86}\text{Sr} \sim 0.7165$. As the estimate of Phanerozoic silicate sediment initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios is based on present-day $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{87}\text{Rb}/^{86}\text{Sr}$ ratios, post-depositional changes in either parameter might result in the estimated initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios differing from the initial $^{87}\text{Sr}/^{86}\text{Sr}$

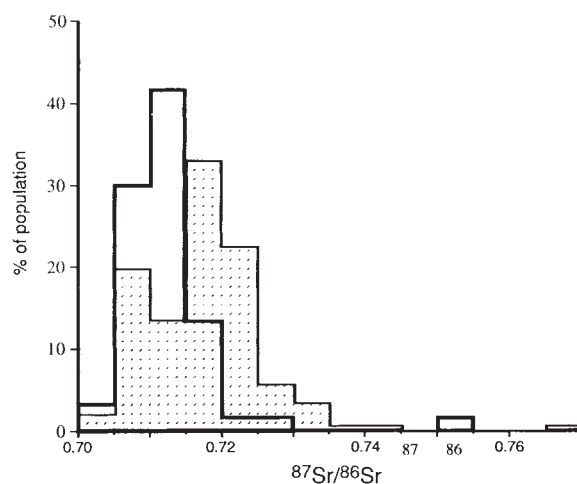


FIG. 3 Histograms of initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of Phanerozoic sediments (unshaded area, thick boundary lines) and of modern detrital silicate fraction in rivers, loess and the oceans (dotted area). Sources for the initial $^{87}\text{Sr}/^{86}\text{Sr}$ of Phanerozoic sediments are the same as in Fig. 2: mean value of 0.7112 ± 0.0024 ($n = 59$). Sources of data for the modern silicate fraction (all published analyses with no weighting by location) are refs 6, 58–66: mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio = 0.7178 ± 0.0014 ($n = 221$). Data from oceans includes only acid-leached samples to remove carbonate. Subsidiary maximum between 0.705 and 0.710 may indicate imperfect leaching or the effects of diagenetic exchange. Errors on means are two times standard errors on data sets.

ratios of the sediments. Diagenetic exchange between clay minerals and carbonate or ocean water may reduce the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of silicate sediment material after deposition, but such exchange is only apparent in the finer clay mineral fractions¹⁶. Alternatively, if the silicate-sediment Rb/Sr ratios were increased by diagenesis a significant time after deposition (perhaps due to loss of Sr in a pore fluid during compaction), then the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of the sediment would be underestimated. A final possibility is that there have been secular changes in detrital silicate $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, and that such material has a substantially higher $^{87}\text{Sr}/^{86}\text{Sr}$ ratio today than it had during most of the Phanerozoic eon. The rapid increase in seawater $^{87}\text{Sr}/^{86}\text{Sr}$ ratios since 40 Myr ago has been ascribed to erosion of high $^{87}\text{Sr}/^{86}\text{Sr}$ detritus from the Himalayas^{17,18}. If the proportions of Sr dissolved from carbonate and silicate rock have remained unchanged, but riverine $^{87}\text{Sr}/^{86}\text{Sr}$ ratios have decreased from ~ 0.7119 today¹⁹ to the ~ 0.710 value estimated by Richter *et al.*¹⁸ for the late Cretaceous–early Tertiary, then the average $^{87}\text{Sr}/^{86}\text{Sr}$ of Phanerozoic silicate detrital material may have been as low as ~ 0.713 , compared with 0.7178 today (Fig. 3). The possible secular variations in both the dissolved riverine Sr flux and its $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic composition are currently the subject of much speculation²⁰. Irrespective of the cause of the difference between the estimated initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the silicate fraction of sediments (without the carbonate component) and silicate detritus, both estimates are substantially lower than the mean $^{87}\text{Sr}/^{86}\text{Sr}$ of the silicate sediment mass. The silicate sediment mass must exchange significant Sr with a low $^{87}\text{Sr}/^{86}\text{Sr}$ reservoir at some point(s) in the sedimentary cycle.

Sr isotope mass balance

The rapid recycling of the global sediment mass (half life ~ 160 Myr (ref. 7) implies that its composition must be near steady state for elements and isotopes with significant fluxes during the recycling process. The concentration of any component (or isotopic ratio of any radiogenic tracer) in a constant-mass, well-mixed reservoir subject to constant input fluxes and with corresponding output fluxes, approaches steady state with an exponential dependence on time, t , as e^{-bt} (ref. 8). The magnitude of the time constant, b , is equal to the ratio of the magnitude of the output flux for that component to the mass of the component in the reservoir. The main loss of Sr from the silicate sediment mass is by weathering to solution (during sedimentary recycling) and by cycling through metamorphic belts, as discussed further below. The Sr flux to solution from silicate rocks may be estimated from the riverine dissolved Sr load (2.9×10^{12} g Sr yr⁻¹) and $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (0.7119) (ref. 19). The riverine dissolved load is derived in part from carbonate with a mean $^{87}\text{Sr}/^{86}\text{Sr} = 0.708$, calculated from the sediment mass–age distribution⁷ and seawater $^{87}\text{Sr}/^{86}\text{Sr}$ variation²¹. If the rest of the dissolved load is derived from weathering of silicate rocks with the same $^{87}\text{Sr}/^{86}\text{Sr}$ as the modern silicate detrital fraction of (0.7178 ± 0.0014 , Fig. 3) then $\sim 40\%$ of the riverine dissolved load is derived by weathering of silicates, implying a flux of 1.15×10^{12} g Sr yr⁻¹. This flux is derived by weathering of silicate sediments, metamorphic rocks and igneous crust but the component from sedimentary rocks is substantial⁴. The exponential time constant for return to Sr isotope steady state in the silicate sediment mass ($\sim 4.5 \times 10^{20}$ g Sr) is $\sim 2.6 \times 10^9$ yr⁻¹ which implies a decay time (shift by a factor of $1/e$ towards equilibrium) of ~ 400 Myr. The Sr isotope composition of the silicate sediment mass must be near steady state, consistent with the uniform initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (Fig. 2b). The Sr lost to solution by weathering must be replaced by Sr from another source. As all the possible sources (mantle, lower crust and the ocean or carbonate reservoirs), have low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, it is probable that the return Sr flux—which must balance this loss to solution—is responsible for the moderation of silicate sediment $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The secular increase in Rb/Sr ratio, suggested by McDermott and Hawkesworth²² to explain Phanerozoic silicate-sedi-

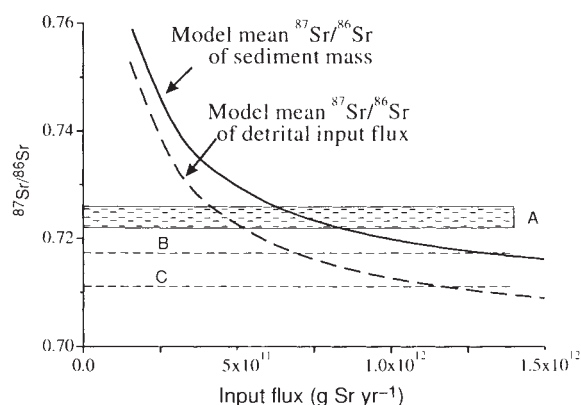


FIG. 4 Average $^{87}\text{Sr}/^{86}\text{Sr}$ composition of silicate sediment mass (4.5×10^{20} g Sr, Rb/Sr = 1.0) calculated for an input of 1×10^{11} g Sr yr⁻¹ from new crust ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7035$) as a function of the mass of Sr exchanged with the ocean/carbonate reservoir ($^{87}\text{Sr}/^{86}\text{Sr} = 0.708$) during recycling of sediment by erosion or through metamorphic belts. Also shown is the mean $^{87}\text{Sr}/^{86}\text{Sr}$ composition of detrital silicate material at the time of deposition if the Sr input from the ocean/carbonate reservoir is added to the detrital flux before sedimentation. Observed range of mean $^{87}\text{Sr}/^{86}\text{Sr}$ of silicate sediment mass (Fig. 2a) (horizontal box A), mean detrital silicate (horizontal dashed line B) and mean initial $^{87}\text{Sr}/^{86}\text{Sr}$ of silicate sediment (Fig. 3) (horizontal dashed line C) shown for comparison.

ment Sr-isotope systematics, is an unlikely explanation as the rapid recycling of the sediment mass will result in a near steady-state composition.

As discussed by Goldstein¹, the low $^{87}\text{Sr}/^{86}\text{Sr}$ reservoirs on Earth are the mantle, lower crust, carbonate sediments and sea water. The Nd isotope evolution of the silicate sediment mass precludes significant addition of new mantle-derived crust, and lower crust is not a plausible source. The additional return Sr flux must therefore be derived from the oceans or carbonate. Figure 4 illustrates the mean $^{87}\text{Sr}/^{86}\text{Sr}$ composition of the silicate sediment mass and the mean $^{87}\text{Sr}/^{86}\text{Sr}$ composition of the input flux to the silicate sediment mass, plotted against the input flux of Sr from the oceanic or carbonate reservoirs assuming constant mass of strontium in the silicate sediment mass. An additional 10^{11} g Sr yr⁻¹, $^{87}\text{Sr}/^{86}\text{Sr} = 0.7035$, is presumed added from new crust. An Rb/Sr ratio of 1.0 has been assumed for the silicate sediment mass. This is intermediate between that of the 0.8 value of Taylor and McLennan⁵ based on a large sedimentary data set and the 0.9 to 1.7 range constrained by the data set in Fig. 2. The latter may be biased to high Rb/Sr ratios by the numerous attempts to date fine-grained shales. Figure 4 illustrates, for example, that the mean $^{87}\text{Sr}/^{86}\text{Sr}$ of a silicate sediment mass (2.24×10^{24} g with 200 p.p.m. Sr) would be maintained at $^{87}\text{Sr}/^{86}\text{Sr} = 0.724$ if 8×10^{11} g Sr yr⁻¹ were lost to solution (or metamorphic belts) and replaced by 10^{11} g Sr yr⁻¹ from new crust and 7×10^{11} g Sr yr⁻¹ from the ocean/carbonate reservoir. Assuming that all the ocean/carbonate flux were added to the detrital silicate material before sedimentation (that is, no Sr was added by diagenesis), the mean $^{87}\text{Sr}/^{86}\text{Sr}$ composition of the detrital component would be 0.7171. To a first approximation, it seems that the Sr isotope evolution of the silicate sediment mass is explained by loss of Sr to solution during weathering and erosion, and its replacement from the oceanic or carbonate reservoirs.

Sr return flux and a model for Sr isotope fluxes

Strontium may be returned to the silicate sediment mass from the ocean water or carbonate rock by (1) reaction of silicate sediment with pore-water or carbonate during diagenesis after deposition, or (2) breakdown of carbonate minerals to silicate minerals (decarbonation reactions) during metamorphism, fol-

lowed by erosive recycling into the sediment mass. Significant Sr transport from carbonate horizons to adjacent silicate rocks by advection and/or diffusion in the fluid phase during metamorphism is not observed²³.

The relative contributions of diagenesis and metamorphic decarbonation reactions to the Sr isotope budget of the silicate sediment mass are poorly resolved. There are two lines of evidence that suggest that significant Sr is transferred from carbonate to silicate minerals during metamorphism. The rapid recycling of the sediment mass and consequent loss of $\sim 6.7 \times 10^{12}$ mol yr^{-1} of Ca + Mg to solution⁴, compared with the magnitude of Ca + Mg in the silicate sediment mass ($\sim 3 \times 10^{21}$ mol, ref. 5) indicates that the Ca + Mg content approaches steady state with an exponential decay time of ~ 450 Myr, similar to that for Sr. The return Ca flux must involve silicate-mineral producing decarbonation reactions during metamorphism, because significant Ca-bearing silicate minerals are not produced during diagenesis. The second line of evidence is the observation that the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of present-day silicate detrital material (0.7178) is substantially lower than the mean $^{87}\text{Sr}/^{86}\text{Sr}$ of the silicate sediment mass (0.7235 ± 0.0035). This requires that at least part of the low-isotope-ratio return Sr flux to silicate sediments is added to detrital silicate before its deposition; this is because Sr isotope interaction between silicate detritus and the oceans is negligible during erosion, transport and deposition⁶. The possible source of low $^{87}\text{Sr}/^{86}\text{Sr}$ detritus is silicate material derived from young exhumed metamorphic rock, in which the average $^{87}\text{Sr}/^{86}\text{Sr}$ of the silicate minerals is reduced by transfer of Sr from carbonate to silicate minerals (by metamorphic decarbonation reactions).

If metamorphism is to be an effective moderator of the Sr isotope ratio of the silicate-sediment mass, then metamorphic rock must predominate in erosional detritus from crystalline crust. Also, metamorphism must be accompanied by relatively rapid erosion of the metamorphic rock to preclude radioactive ^{87}Sr production. Such rapid recycling is implied by the metamorphic pressure-temperature-time arrays from subduction and collisional settings, which require that metamorphic belts are uplifted and eroded within 30 to 60 Myr of their formation²⁴ and given mass estimates of sediments produced by unroofing of recent metamorphic belts²⁵. The low initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of S-type granites (0.710 to 0.720) produced by melting of sediments in high-temperature metamorphic belts provides confirmation of the significance of Sr isotope exchange during metamorphism²⁶. The flux of sediment through metamorphic belts is very poorly constrained. A minimum estimate may be derived from the mass of sediment in the deep oceans and oceanic margins (7×10^{23} g (ref. 7), which is recycled through accretionary prisms above subduction zones at $\sim 3 \times 10^{15}$ g yr^{-1} . Sediment metamorphosed in collisional Himalayan-type belts and extensional continental regimes would augment this flux. If 4×10^{15} g yr^{-1} of sediment is metamorphosed this represents $\sim 40\%$ of sediment recycling.

Figure 5 illustrates a self-consistent model for Sr isotope fluxes which satisfies the following constraints: (1) a mean detrital silicate $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7178; (2) recycling of the sediment mass of 2.24×10^{24} g at 9.6×10^{15} g yr^{-1} (ref. 7), with 4×10^{15} g yr^{-1} of this recycled through metamorphic belts and the rest resedimented; (3) loss of 11.5×10^{11} g Sr yr^{-1} to solution during erosive recycling of both silicate sediment and crystalline metamorphic rock; (4) an input of 10^{11} of Sr from new crust ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7035$), (5) an Rb/Sr ratio for the silicate sediment mass of 1.0 and (6) the diagenetic transfer of 5×10^{11} g Sr yr^{-1} ($^{87}\text{Sr}/^{86}\text{Sr} = 0.708$) from the ocean/carbonate reservoir to the silicate sediment mass. It is presumed that metamorphic belts contain constant mass, and that material is cycled through metamorphic belts rapidly compared with radioactive decay (time constant $< \sim 60$ Myr). The mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the silicate sediment mass and of metamorphic rocks, the proportion of the Sr returned to the silicate sediment mass in erosively recycled

sediment, and the Sr lost to solution from metamorphic rocks are calculated from the Sr and Sr isotope mass-balance equations.

The principal uncertainty in Fig. 5 is the relative proportions of Sr transferred to the silicate sediment mass by diagenesis, and that transferred by decarbonation reactions in metamorphic belts. The relative partitioning is irrelevant to the Sr isotope budget of the silicate sediment mass; it is however, significant both for the magnitude and location of CO_2 production by decarbonation reactions (in metamorphic belts), and for the magnitude of several of the model parameters, including the Sr isotope composition of silicate metamorphic rocks and their erosional detritus. Figure 5 was constructed on the basis that the amount of Sr transfer during metamorphism (5.5×10^{11} g Sr yr^{-1}) would be associated with production of sufficient CO_2 to balance CO_2 adsorption by silicate weathering⁴, with a corresponding flux of Ca + Mg from carbonate to silicate minerals. A Sr/(Ca + Mg) weight ratio of 2×10^{-3} for carbonate (ref. 27) is used in the calculations. The additional 5×10^{11} g Sr yr^{-1} needed to maintain the Sr content of the silicate sediment mass is presumed to be transferred during diagenesis. With these assumptions, the silicate sediment Sr isotope budget is satisfied by an input of 6.6×10^{11} g Sr yr^{-1} (with a $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7135) from metamorphic belts, a loss to solution from recycling of sediment of 4.6×10^{11} g Sr yr^{-1} , and a loss to solution from erosive recycling of metamorphic rock of 6.9×10^{11} g Sr yr^{-1} . 10.5×10^{11} g Sr yr^{-1} are transferred from the oceanic or carbonate reservoirs to silicate material, but 2.8×10^{11} g Sr yr^{-1} of this are lost to solution during the erosive recycling of metamorphic rock. The predicted mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the silicate sediment mass is 0.7242.

If significantly more Sr were transferred by diagenesis and less by metamorphism (compared to the model shown in Fig. 5), the estimate of the mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the silicate sediment mass is reduced to values less than ~ 0.720 and also lower than those observed (Fig. 2a). If little Sr were transferred by diagenesis, then the estimated CO_2 production by decarbonation in metamorphic belts is increased to 13×10^{12} mol yr^{-1} , double the estimate of adsorption by silicate weathering⁴. We emphasize that current uncertainties in many of the model parameters, particularly reservoir sizes, compositions, recycling rates and the mean carbonate Sr/(Ca + Mg) ratio, preclude a rigorous estimate of error on the model. The best-fit solution (Fig. 5) requires significant Sr transfer by diagenesis and metamorphism; it is consistent with metamorphic decarbonation reactions producing most of the CO_2 required to balance silicate weathering.

Implications

The variation of oceanic $^{87}\text{Sr}/^{86}\text{Sr}$ ratios during the Phanerozoic eon provides a finely resolved record which must reflect a balance between inputs of Sr of continental derivation and Sr input by hydrothermal circulation through the oceanic crust. Attempts to couple the Phanerozoic seawater $^{87}\text{Sr}/^{86}\text{Sr}$ curve to carbon-cycle climatic models have met with limited success^{28,29}, probably because the decarbonation and Sr-return processes are incorrectly coupled into the models. Seawater Sr isotope variations are not reproduced by simple relationships between seafloor spreading rates, sea levels, atmospheric CO_2 levels and weathering rates. In order to make any match between model and theory, arbitrary forcing of the continental Sr isotope weathering flux is required. Berner and Rye²⁹ have produced a partial solution by assuming that the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the weathering flux of silicate-derived Sr is a function of sea level. This produces a general match between their model seawater $^{87}\text{Sr}/^{86}\text{Sr}$ curve and observed values for periodicities of ~ 200 Myr or more, but it does not produce the marked variations in seawater $^{87}\text{Sr}/^{86}\text{Sr}$ on the ~ 50 Myr timescale. It seems probable that the 50-Myr variations relate to individual orogenic episodes, and that the marked variations in $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (0.7160 to 0.709) of silicate-derived Sr demanded by Berner and Rye's models arises from rapidly varying uplift of silicate metamorphic rock, the stron-

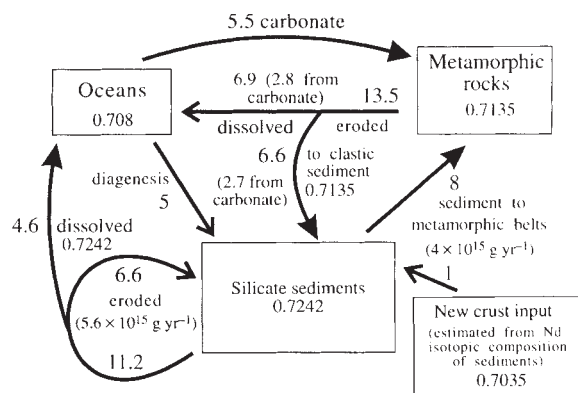


FIG. 5 Self-consistent Sr isotope flux model for the silicate sediment mass. The numbers on the arrows represent the Sr fluxes in units of $10^{11} \text{ g yr}^{-1}$; the numbers in each box represent the mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio. The model is calculated on the premise that the mean $^{87}\text{Sr}/^{86}\text{Sr}$ of detrital silicate is 0.7178 (Fig. 4), the riverine dissolved Sr load ($2.9 \times 10^{12} \text{ g yr}^{-1}$ (ref. 19) is derived from silicate with $^{87}\text{Sr}/^{86}\text{Sr} = 0.7178$ and carbonate with $^{87}\text{Sr}/^{86}\text{Sr} = 0.708$, and the silicate sediment mass ($2.24 \times 10^{24} \text{ g}$, 200 p.p.m. Sr) is recycled at $9.6 \times 10^{15} \text{ g yr}^{-1}$ and contains $4.5 \times 10^{20} \text{ g}$ of Sr with an Rb/Sr ratio = 1.0. Added from new crust is $10^{11} \text{ g Sr yr}^{-1}$ ($^{87}\text{Sr}/^{86}\text{Sr} = 0.7035$). It is presumed that Sr lost to solution by weathering is replaced by diagenesis, and decarbonation reactions in metamorphic belts and metamorphic rock is rapidly recycled into the sediment mass.

tium isotope of which has been reduced by decarbonation reactions. If the Sr isotope composition of silicate-derived Sr is significantly moderated by metamorphism, then the seawater $^{87}\text{Sr}/^{86}\text{Sr}$ ratio will be partly controlled by the processes of decarbonation, uplift and erosion; these processes are coupled in a complex manner to the other processes affecting seawater $^{87}\text{Sr}/^{86}\text{Sr}$ variations. The coupling will include a variable time-lag between deposition and metamorphism. Whether changes in metamorphic rates exert sufficient control on CO_2 fluxes to cause significant climate change is not known³⁰. It should be noted that release of CO_2 during metamorphism will be essentially instantaneous, related to the compaction time of porous metamorphic rock, whereas release of low $^{87}\text{Sr}/^{86}\text{Sr}$ silicate detritus will have a ~ 10 Myr time lag related to uplift and erosive processes.

The other uncertainty in coupling metamorphic processes into CO_2 and Sr isotope flux models is the location and precise cause of decarbonation reactions. Carbonate minerals will break down in significant quantities when heated above $\sim 600^\circ\text{C}$; however, it has been suggested that extensive decarbonation in several terrains relates to infiltration of substantial volumes of water-rich fluids at more moderate temperatures ($400\text{--}600^\circ\text{C}$) (ref. 31). If so, the availability of a water-rich fluid and hydrothermal flow during the metamorphic event may be at least as important as tectonic rates in moderating both CO_2 release and Sr isotope exchange. The magnitude of fluid flow in metamorphic events is uncertain and controversial^{32,33}. For example, estimates of time-integrated fluid fluxes during the Acadian metamorphic events in Maine and Vermont are $\sim 5 \times 10^3 \text{ m}^3 \text{ m}^{-2}$ of a fluid with 5 to 10 mol% CO_2 (refs 34, 35). If this flux was characteristic of metamorphism, and the affected flow paths of ~ 10 km length, metamorphism of $4 \times 10^{15} \text{ g yr}^{-1}$ of sediment would produce up to $4 \times 10^{12} \text{ mol yr}^{-1}$ of CO_2 . This is of similar order to the CO_2 flux estimate of $6.7 \times 10^{12} \text{ mol yr}^{-1}$ based on the riverine dissolved load. The dominant location and mechanism of CO_2 production also has implications for attempts to explain apparent discrepancies in present-day atmospheric CO_2 fluxes³⁶. Of particular importance is whether metamorphically derived CO_2 is released directly to the atmosphere or to the oceans³⁷.

Discussion

The $^{87}\text{Sr}/^{86}\text{Sr}$ composition of the silicate sediment mass is moderated both by an input of $\sim 7.7 \times 10^{11} \text{ g yr}^{-1}$ of Sr from the oceanic and carbonate reservoirs and by $\sim 10^{11} \text{ g yr}^{-1}$ of Sr from juvenile crust, which together balance the Sr flux lost to solution during weathering and erosion. The observed mean $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of the silicate sediment mass and present-day detrital silicate material are most closely modelled if about equal amounts of Sr from the oceans or carbonate are added during diagenesis and metamorphism. The model predicts that CO_2 production in metamorphic belts is complementary to the flux of CO_2 adsorbed by weathering of silicate rocks estimated from riverine Ca + Mg loads. This suggests that a significant amount of decarbonation takes place in the crust and therefore that mantle CO_2 degassing is not the prime source of CO_2 , which is consistent with some estimates of volcanic CO_2 fluxes (see, for example, ref. 38). Models that attempt to relate tectonic activity, sea level, weathering and climate to atmospheric CO_2 levels and the seawater Sr isotope variation should take into account the influence of metamorphic processes on decarbonation and potential release of low $^{87}\text{Sr}/^{86}\text{Sr}$ silicate to the sediment mass. □

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Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution

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The structure of the MAP kinase ERK2, a ubiquitous protein kinase target for regulation by Ras and Raf, has been solved in its unphosphorylated low-activity conformation to a resolution of 2.3 Å. The two domains of unphosphorylated ERK2 are farther apart than in the active conformation of cAMP-dependent protein kinase and the peptide-binding site is blocked by tyrosine 185, one of the two residues that are phosphorylated in the active enzyme. Activation of ERK2 is thus likely to involve both global and local conformational changes.

THE MAP kinases ERK1 and ERK2 have been widely studied as ubiquitous, growth-factor-activated, tyrosine-phosphorylated enzymes^{1–3}. These enzymes phosphorylate many well studied regulatory proteins, including transcription factors^{4,5}, membrane proteins^{6,7}, cytoskeletal elements^{8,9}, and other protein kinases^{10,11}. The MAP kinases are activated by tyrosine kinase receptors, G-protein-linked receptors and protein kinase C-dependent pathways. The tyrosine kinase pathways activate ERKs through a Ras/Raf-dependent cascade^{12,13}; the MAP kinases are major targets of Ras/Raf¹⁴, and may be the primary disseminators of the regulatory signals transmitted by these proto-oncogene products. Because of their function in signal transduction cascades and their pleiotropic regulatory properties, it has been suggested that the ERKs are potential targets for chemotherapeutic intervention in pathological states ranging from cancer to Alzheimer's disease^{15–17}.

ERK2 achieves maximum activity only when both Tyr 185 and Thr 183 are phosphorylated through the action of MEK^{3,18,19}, a dual specificity kinase. The singly phosphorylated forms of ERK2 have less than 1% of the activity of the fully phosphorylated enzyme²⁰. Substoichiometric autophosphorylation also occurs on Tyr 185, giving rise to low levels of activity *in vitro*^{21,22}. The enzyme is almost completely inactivated by the serine/threonine phosphatase 2A, the tyrosine phosphatase CD45 (refs 3, 23), and a VH1-related dual-specificity phosphatase^{24–26}. ERK2 expressed in *Escherichia coli* with a hexa-His tag incorporated at the N terminus is unphosphorylated and, as a result, has low activity. On incubation with its activator MEK, however, this ERK2 achieves the same specific activity as enzyme purified from mammalian cells²⁰. We report here the structure of this unphosphorylated recombinant form of ERK2 at 2.3 Å resolution.

Structure determination

Crystals of ERK2 were grown from polyethylene glycol–

ammonium sulphate mixtures²⁷. Multiple isomorphous replacement (MIR) and real-space molecular replacement methods were used in the structure solution as described in Table 1 legend. It was not possible to solve the structure by molecular replacement from the active conformation of the cyclic AMP-dependent protein kinase (cAPK), with which ERK2 shares 23% sequence identity, because no translation function solution could be found. However, the larger C-terminal domain of cAPK could be superimposed on the MIR electron density map, which aided in subsequent model building and refinement.

Structure description

The main-chain carbonyl atoms and branched side-chain atoms are visible from residues 2–354 in the electron density map (Fig. 2a). The hexa-His tag is disordered, with atomic B-factors²⁸ of ~100 Å², and is visible only when low-order (20 Å) terms are included in the Fourier synthesis. Four residues (355–358) are disordered at the C terminus, and the phosphate anchor ribbon²⁹, which contains the conserved Gly-X-Gly-X-X-Gly sequence³⁰ (strands β 1 and β 2 of the N-terminal domain core β -sheet), is flexible, with an average B-factor of 60 Å². The overall topology of the molecule (Fig. 3a) is similar to those of cAPK and cyclin-dependent kinase 2 (CDK2)^{29,31}.

The structure of ERK2 spans the conserved protein kinase homology region³² and has N- and C-terminal extensions that lie on the surface of the molecule (Fig. 3b). Within the protein kinase homology region, ERK2 has a unique insertion, relative to protein kinases outside the MAP kinase family, in the L₆ linker (Figs 1 and 3b). Like CDK2, ERK2 is missing the B helix present in cAPK²⁹ (Fig. 3a, c). In the C-terminal domain, ERK2 has an insertion after the helix G (Figs 1 and 3a–c) that is common to all members of the CDC2 α family of the kinase family³³. This insertion consists of two helices (α 1L₁₄ and α 2L₁₄) that contact the phosphorylation 'lip' (L₁₂, containing the phosphorylation sites Thr 183 and Tyr 185; Fig. 3a, c).

The N-terminal extension of ERK2 (residues 1–21), is formed into a β -ribbon between residues Ala 5 and Glu 18 at the top of

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TABLE 1 Statistics for X-ray data collection, phase determination and refinement

Diffraction data												
Derivative	Concentration (mM)	Soak time (h)	$d_{\min}$ (Å)	No. of measurements	Unique data (% completeness)							R_{merge}^* (%)
Native			2.3	62,796	16,950 (97)							5.1
Thimerosal	0.2	12	2.5	57,169	13,690 (98)							5.5
KAu(CN) ₂	5.0	96	3.0	53,108	7,660 (91)							4.4
YbAc ₂	10.0	48	2.5	36,538	12,350 (89)							5.7
PHMB	0.2	24	2.5	34,681	11,320 (81)							8.0
Mg-ATP	10.0	6	2.5	45,867	12,250 (90)							5.7
Mg-AMP-PNP	5.0	6	2.5	27,191	12,542 (89)							5.9
MIR phasing												
Derivative	R_f † (%)	No. of sites	R_c ‡	F_o/E_s at each resolution bin (Å)								
				(7.5)	(6.3)	(5.3)	(4.6)	(4.1)	(3.6)	(3.5)	(3.0)	Total
Thimerosal	28	4	0.68	2.38	2.19	3.00	1.83	1.35	1.52	1.54	1.77	1.95
KAu(CN) ₂	14	4	0.57	1.87	1.87	1.57	1.31	1.11	1.09	1.29	1.49	1.45
YbAc ₂	15	1	0.78	0.37	0.40	0.40	0.35	0.30	0.30	0.26	0.25	0.35
PHMB	30	5	0.73	1.54	1.79	1.89	1.54	1.53	1.22	1.16	1.64	1.54
FOM				0.86	0.87	0.89	0.84	0.81	0.74	0.70	0.65	0.75
Refinement												
	Resolution (Å)	Number of reflections ($F > 2\sigma$)		Total number of atoms		R -factor¶ (%)	R.m.s. deviation $\Delta(\text{bond})$ (Å)			$\Delta(\text{bond})$ (°)		
Native	6.0 – 2.3	13,272		2959		17.2	0.015			2.70		
	20.0 – 2.3	15,524		3079		18.8	0.018			2.90		
Mg-ATP	6.0 – 2.5	10,525		2991		16.5	0.016			2.80		

Expression and crystallization. A construct designed to express ERK2 with His₆ at its N terminus was subcloned into the bacterial expression vector NpT7-5 and expressed and purified as described²⁷. Large crystals were obtained by combining 6 mg ml⁻¹ ERK2 solutions with 18% PEG 8000, 0.2 M (NH₄)₂SO₄, 50 mM MES, 5 mM dithiothreitol, 0.1 mM Na₂S₂O₃, pH 5.9 at 21°. The crystals belong to space group *P*2₁ and have unit cell dimensions of $a = 49.32$ Å, $b = 71.42$ Å, $c = 61.25$ Å, $\beta = 109.75^\circ$. **Diffraction data.** These were collected at room temperature with dual multiwire area detectors of Xuong-Hamlin design with graphite monochromated CuK α X-rays generated by a Rigaku RU200 rotating-anode source operated at 50 kV, 100 mA. Images were recorded as 0.1° oscillation frames. Integrated intensities with anomalous dispersion were scaled and merged with software by Howard and Nielsen⁴⁵. **Heavy-atom derivatives and MIR analysis.** Crystals were stabilized in 22% PEG 8000, 0.2 M (NH₄)₂SO₄, 0.1 mM Na₂S₂O₃, pH 5.9, then incubated for 12–96 h in individual heavy-atom compounds. Derivatized crystals were incubated in heavy-atom-free stabilizing solution for 0.5 h before data collection. The positions of the Hg atoms in the thimerosal derivative were determined from the difference Patterson function. Positions for *p*-hydroxymercuribenzoate (PHMB), KAu(CN)₂, and Yb acetate were found by difference Fourier synthesis using solvent-flattened single isomorphous replacement phases based on the thimerosal derivative. Heavy-atom parameters were refined with MIRAS (multiple isomorphous replacement with anomalous scattering) phases using PROTEIN⁴⁶. The heavy-atom parameters were further refined and minor sites located using partial structure-derived phases as restraints. **Solvent flattening.** Initial electron density maps from four derivatives were calculated at 3.0 Å. Phases were modified using an iterative solvent flattening procedure⁴⁷. The solvent volume was set at 35%; 10 cycles of solvent flattening were carried out to give an electron density with a figure of merit of 0.85. **Model building and refinement.** Real space search routines in PROTEIN were used to locate the cAPK model in the solvent-flattened MIR Fourier map. The solution assisted with identification and model building of the larger C-terminal domain, but not the small domain. The polypeptide chain was fitted to the electron density with programs FRODO⁴⁸ and O⁴⁹ using the main-chain database and side-chain rotamer libraries in O. The initial model was then refined by the conventional positional refinement protocols in XPLOR⁵⁰, gradually extending the resolution to 2.5 Å. Simulated annealing was then used at 2.5 and 2.3 Å resolution using reflections with $|F| > 2\sigma$. The loops between residues 30–36, 55–60 and 173–186 were refitted in annealed omit maps. Residues 2–16, 24–35, 42–48, 58–60 and 328–336 had B-factors > 50 Å². 120 water molecules were identified and added in the last refinement cycle.

* $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum I_i \times 100$, where I_i is the intensity of an individual measurement, and $\langle I \rangle$ is the mean intensity of this reflection.

† $R_f = \sum ||F_{\text{ph}}| - |F_{\text{p}}|| / \sum |F_{\text{p}}| \times 100$, where $|F_{\text{ph}}|$ and $|F_{\text{p}}|$ are protein and heavy-atom derivative structure factor amplitudes, respectively.

‡ $R_c = \sum ||F_{\text{ph}} \pm F_{\text{p}}| - F_{\text{calc}}| / \sum |F_{\text{ph}} \pm F_{\text{p}}|$ for centric reflections.

§ F_o/E is the phasing power, where F_o is the mean amplitude of heavy-atom structure factor, and E is the r.m.s. lack-of-closure error.

|| FOM, figure of merit.

¶ $R\text{-factor} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}| \times 100$, where $|F_{\text{obs}}|$ and $|F_{\text{calc}}|$ are the observed and calculated structure factor amplitudes respectively.

the molecule (Fig. 3a, b). The C-terminal extension (residues 315–358) begins at residue Tyr 315 (Fig. 3b). Between residues Asp 316 and Pro 337, the polypeptide is extended and forms part of the domain interface; residues Lys 338 to Glu 353 form an α -helix (α L₁₆) within the body of the N-terminal domain. The functional significance of the placement of the N- and C-terminal extensions is unknown. However, these sequences have no structural homology, or sequence homology, to extensions in cAPK (Fig. 3b); and, as is apparent from Fig. 3b, the N- and C-terminal extensions of ERK2 and cAPK have exchanged places.

Domain separation

The α atoms within individual domains of ERK2 superimpose well with corresponding atoms in cAPK. The C-terminal domains (residues 109–314) superimpose with an r.m.s. deviation for α atoms of 1.8 Å; a single systematic difference is found in strands β 7 and β 8 (Fig. 3a, c) in the domain interface.

In the N-terminal domain, the central strands β 3, β 4 and β 5 superimpose with an r.m.s. deviation for α atoms of 1.7 Å. However, the position of the C helix and strands β 1 and β 2 of the phosphate anchor ribbon²⁹ differ and contribute to an open active site in ERK2.

The overall r.m.s. superposition of α atoms of unphosphorylated ERK2 and active cAPK is 3.1 Å, significantly larger than the deviation of the individual domains. The discrepancy is a result of a rotation of the N-terminal domain of low activity ERK2 by 17° relative to that of active cAPK (Fig. 3c), about an axis passing between the two domains, which leaves the active site of ERK2 more open.

In ERK2 the active site is held open by the interaction of the C helix and the conserved glycine of the Asp-Phe-Gly sequence (residues 165–167, subdomain VII; ref. 32). In the N-terminal domain, the domain interface consists of the C helix and the linker between the C helix and β 4 (L₅). In the C-terminal

domain, the domain interface consists of the E helix and an arched β -ribbon comprised of strands $\beta 6$, $\beta 7$, $\beta 8$ and $\beta 9$. The ribbon is broken by a 3_{10} turn between $\beta 6$ and $\beta 7$, and by the Asp-Phe-Gly sequence between $\beta 8$ and $\beta 9$, forming the active site. The N terminus of the C helix lies on top (Fig. 3d) of Gly 167 (Gly 186) (numbers in parentheses refer to the analogous position in cAPK) of the Asp-Phe-Gly sequence. The domains are knitted together toward the back in Fig. 3d, where the polypeptide crosses between the two domains. Superposition of ERK2 and cAPK reveals few conformational differences where the interface is knitted together, but near the N terminus of the C helix (Fig. 3d) large differences are apparent. In ERK2 Arg 65 (His 87) in the C helix points toward the active site, and Gly 167 interdigitates with Arg 65 and Gln 64 (Glu 86). By contrast, in cAPK residue His 87 in the C helix faces towards the phosphorylation site, and Gly 186 interdigitates His 87 and Thr 88. Thus, ERK2 exhibits a different interdigitation of Gly 167 with residues in the C helix, which promotes an open conformation of the active site. Further, the position of the phosphate anchor ribbon contributes to the accessibility of the catalytic site, as already noted (Figs 3c and 4a, b). Given the special contacts that hold the two domains apart, the large domain separation observed in ERK2 is unlikely to be due to crystal contacts, as

was proposed for an open conformation observed in a cAPK-peptide complex³⁴.

Catalytic residues

By analogy with cAPK and the conserved residues in the protein kinase family³⁴, we can speculate as to the identities of the catalytic residues. Within each domain, catalytic residues have interactions quite similar to those in cAPK. For example, in the C-terminal domain the putative catalytic base Asp 147 (Asp 166) and the Mg^{2+} ligand Asp 165 (Asp 184)^{30,35} take up positions nearly identical to those in cAPK; and the hydrogen-bonding network between Asp 165, Asn 152 (Asn 171), Asp 147, Lys 149 (Lys 168)³⁵ and Thr 188 (Thr 201) in ERK2 is similar to that in cAPK. In the N-terminal domain, Lys 52, an essential phosphate-binding residue^{20,30}, is in a position corresponding to Lys 72 of cAPK and forms an ion pair with Glu 69 (Glu 91), as in cAPK. However, the catalytic residues are misaligned across the domain interface (Fig. 4a). Lys 52N ζ is 12.4 Å away from Asp 147O ϵ 2, compared with 7.8 Å in cAPK. The phosphate anchor ribbon (P) and the phosphorylation lip are 4 Å farther apart in ERK2 (Figs 4a, b). These differences may contribute to the low activity of this conformation of ERK2. In the structure of inactive CDK2, no difference in domain separation relative

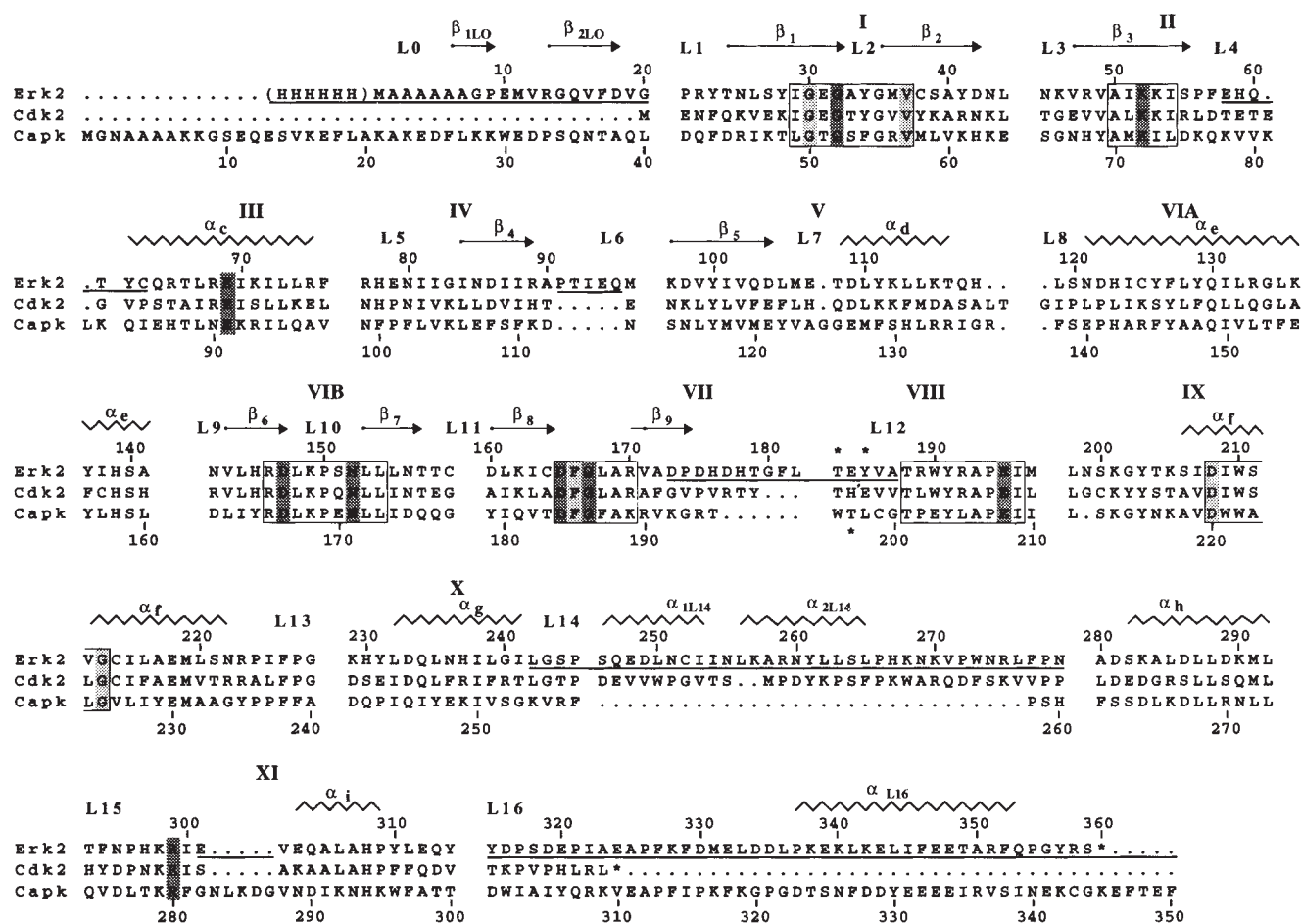


FIG. 1 Structure-based sequence alignment of rat ERK2⁵², human CDK2⁵³ and mouse cAPK⁵⁴. ERK2 shares 38% sequence identity with CDK2 and 23% with cAPK. Subdomains are labelled according to ref. 32. Secondary structure elements in ERK2 are indicated (nomenclature as for Fig. 3a). Shadows highlight invariant (dark) and semi-invariant

(medium) residues in the protein kinase family. Underlined sequences denote structural divergence. Both ERK2 and cAPK numbering are shown. Phosphorylation sites in the phosphorylation lip are denoted by an asterisk.

to the active conformation of cAPK is observed. Instead, the catalytic residues are misaligned owing to local conformational differences in the Asp-Phe-Gly sequence³¹.

Peptide substrate specificity

The peptide substrate binding sites identified in cAPK³⁶ lie mainly in the C-terminal domain, which superimposes well with ERK2. Thus, although the structure of ERK2 has been solved in the unbound state, it is possible to understand some aspects of the difference in specificity by comparing the structures of the two enzymes. Members of the MAP kinase family phosphorylate substrates containing a proline at the P+1 position³⁷, as do members of the CDC2 family. On the other hand, substrates for cAPK and many other kinases contain a hydrophobic residue at the P+1 site. The P+1 residue (Ile 22) of the protein kinase inhibitor (PKI) co-crystallized as a complex with cAPK, binds in a hydrophobic pocket formed by residues Leu 198 to Leu 205, including Gly 200. Of the residues unique to the MAP kinases and the CDC2 family, two residues, Arg 192 (Leu 205) and Ala 187 (Gly 200) (valine in CDK2), are found in the P+1 site. These residues, Arg 192 and Ala 187, as well as Arg 189 (Pro 202), occlude the P+1 pocket (Fig. 4c) present in cAPK. Gly 200 of cAPK is highly conserved outside the MAP kinase and CDC2 family and has (ϕ , ψ) angles in cAPK that are inaccessible to non-glycine residues; thus, this residue is probably important to substrate binding in a number of kinases. The difference in packing of the G helix and the identity of residues Tyr 231, Gln 234 and Leu 235 in the G helix appear to be important to the formation of this pocket also.

A second salient difference between ERK2 and cAPK can be found at the P-3 site. cAPK recognizes arginine in positions P-2 and P-3, whereas good substrates for ERK2 possess hydrophobic residues at P-3^{38,39}. At P-3 in cAPK, the side chain of the inhibitor binds to the ribose of ATP in a ternary complex. In ERK2, Lys 112 interacts with the ribose ring (see below). Lys 112, then, may contribute to the specificity of ERK2 for non-charged residues at P-3. In general, peptide substrates do not bind as well to ERK2 as protein substrates, suggesting the presence of an extended region of interaction with the substrate. Two segments of the structure, the phosphorylation lip (L₁₂) and the large insertion (α 1L₁₄ and α 2L₁₄) contribute to a difference in overall topology of the peptide-binding region relative to cAPK, and may be involved in substrate recognition.

Phosphorylation sites

The phosphorylation sites Thr 183 and Tyr 185 are part of linker L₁₂, the loop between protein kinase subdomains VII and VIII. The L₁₂ 'lip' is six residues longer in ERK2 than in cAPK and three residues longer than in CDK2 (Fig. 3d). The lip is well-ordered, and takes up a conformation completely distinct from the corresponding residues in cAPK or CDK2. The residues that are phosphorylated by MEK, Thr 183 and Tyr 185, are clearly visible in the electron density map (Fig. 2a). Thr 183 is on the surface of the molecule and well-ordered, and the Thr 183 O γ forms a hydrogen bond with the main-chain Gly 180 carbonyl oxygen, also in the lip. Tyr 185 is buried in a largely hydrophobic pocket comprising residues Val 186, Ile 196, Gly 202 and Ile 207, and the aliphatic part of Arg 192. The hydroxyl group on

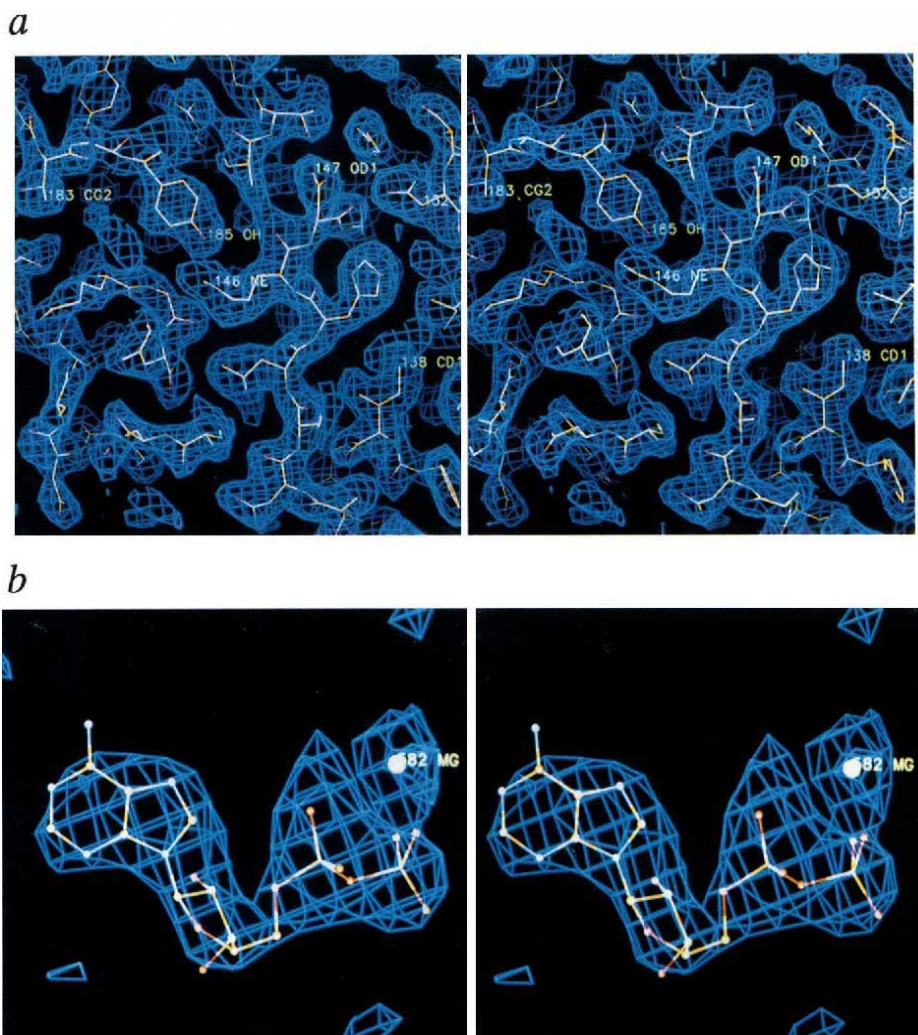
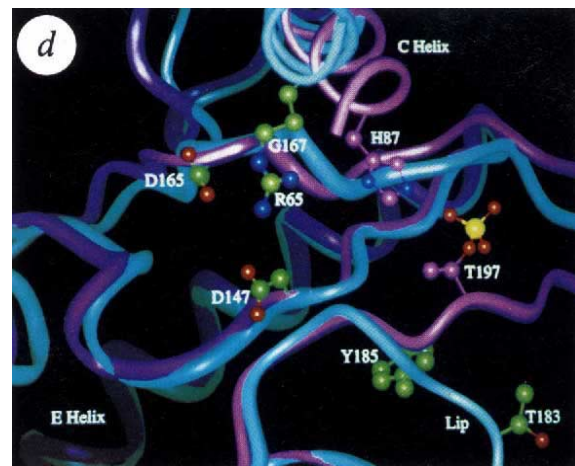
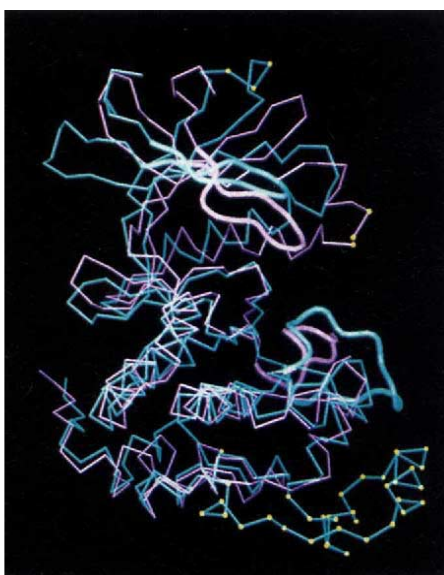
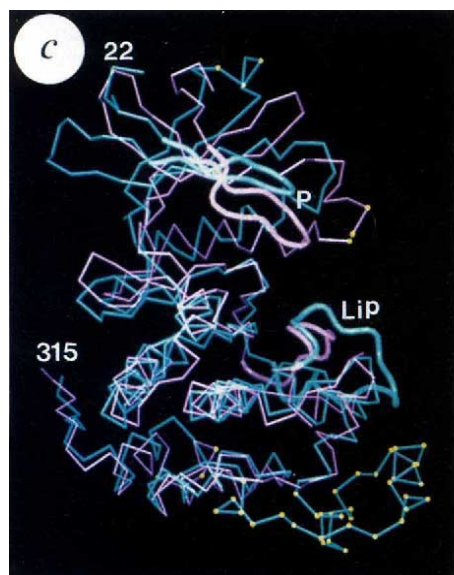
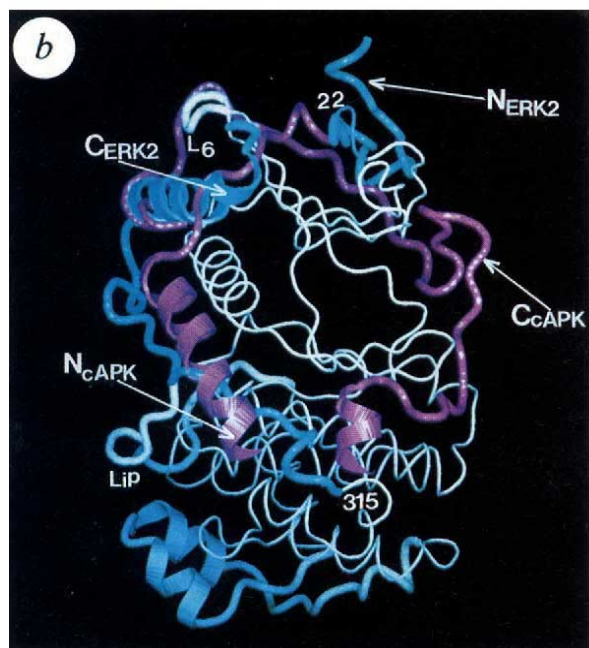
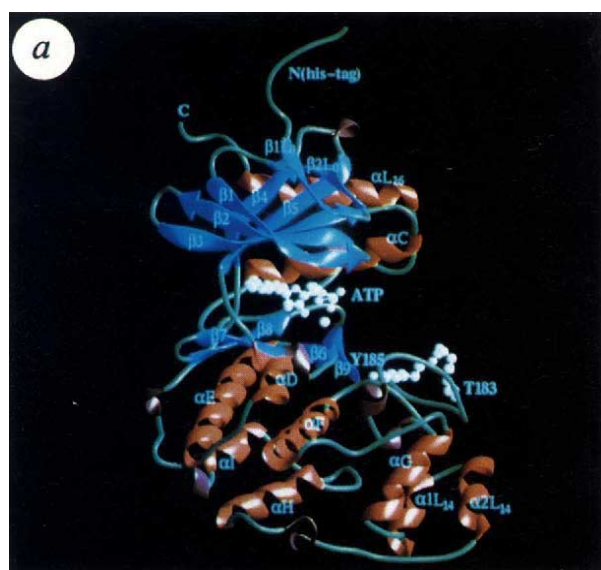


FIG. 2 a, Stereo view of the electron density map contoured at 1.2σ near the β 6 strand and Tyr 185 in the phosphorylation lip. The Fourier map was calculated with model-derived phases and coefficients $2|F_{\text{obs}}| - |F_{\text{calc}}|$ for data between 6 and 2.3 Å. b, Difference Fourier map calculated with native model-derived phases and coefficients $|F_{\text{O}_{\text{MgATP}}}| - |F_{\text{O}_{\text{Native}}}|$ to the diffraction limit of 2.5 Å and contoured at 3.0σ . The γ -phosphate of ATP is disordered and is omitted from the model. Only the high-affinity metal-binding mode³⁵ is observed. The complex was prepared by incubating the native crystal with 2 mM MgCl_2 , 10 mM ATP, 50 mM Tris, pH 7.0 in stabilizing solution for 6 h. Crystals incubated for longer periods or in higher concentrations of ATP do not diffract. Co-crystallization trials have not succeeded.



Tyr 185 is hydrogen-bonded to the guanidinium group of Arg 146(Arg 165) (Fig. 4d).

Kinetic analysis of the rate of incorporation of phosphate into tyrosine and threonine of ERK2 by the action of MEK suggests that the enzyme is phosphorylated first on tyrosine. Tyr 185 is buried in the structure, and thus it is apparent that the phosphorylation lip must have a different conformation entirely when it is bound to MEK. Furthermore, steric constraints suggest that the interactions of both Thr 183 and Tyr 185 observed in this structure are likely to be lost on phosphorylation. Glu 184 corresponds to the constitutive phosphorylation site of cAPK and makes few protein contacts.

The position of the main chain and side chain of Tyr 185 are interchanged relative to cAPK. The neighbouring residues Val 186 and Ala 187 of ERK2 occupy locations similar to those of the P + 1 and P + 2 sites of the peptide substrate (PKI) bound to cAPK (Fig. 4c). Thus, the phosphorylation lip between Tyr 185 and Ala 187 in ERK2 may interfere sterically with substrate binding. It is reasonable to propose that the phosphorylation of Tyr 185 causes its side chain to exchange places with its

FIG. 3 a, Ribbon diagram of ERK2 drawn by RIBBONS⁵¹. The α -helices are red, β -strands blue, 3_{10} helices purple and connecting coils cyan. The Mg-ATP and Thr 183-Tyr 185 side-chain atoms are represented with white space-filling models (γ -phosphate is omitted). Helices and β -ribbons are labelled according to ref. 29. Insertions or linkers (L) are labelled after ref. 31: $\beta 1L_0$ stands for the first strand in the N-terminal linker region. b, ERK2 and cAPK viewed from the back of a. The body of the kinase cores, corresponding to Arg 22 to Tyr 315 of ERK2, is white, N- and C-terminal extensions of ERK2 (labelled N_{ERK2} and C_{ERK2}) are cyan, N- and C-terminal extensions of cAPK (labelled N_{cAPK} and C_{cAPK}) are magenta. The loop containing the phosphorylation sites is labelled 'Lip'; L_6 is a unique insertion of ERK2 in the N-terminal domain. The site occupied by Phe 327 and Phe 329 in ERK2 is filled by Trp 30 in the A helix of cAPK, a residue conserved in a number of kinases. c, Stereo diagram of the α -carbon trace of the kinase core of ERK2 and cAPK showing the difference in domain separation (17°) between two molecules. The C-terminal domains were superimposed (same orientation as in a). ERK2 is in cyan, cAPK in magenta, inserted residues are shown as yellow spheres. The phosphate anchor ribbon (P) and phosphorylation lip (Lip) are shown in tubes. d, Domain interface of ERK2 (cyan) and cAPK (magenta) shows the contacts between the C helix and the glycine in the Asp-Phe-Gly sequence. The side chains of Arg 65(His 87) of ERK2 and His 87 of cAPK lie on opposite sides of Gly 167(Gly 186).

main chain, so that the main chain of these three residues will assume a conformation similar to that of active cAPK and thus permit substrate to bind.

ATP-binding mode

By incubating crystals of ERK2 for 6 h or less in Mg-ATP or

in the non-hydrolysable ATP analogue AMP-PNP, we were able to obtain data for nucleotide-bound forms of ERK2. Mg-ATP and Mg-AMP-PNP (not shown) bind similarly. The electron density for the adenine and ribose ring is well-ordered (Fig. 2*b*). The α - and β -phosphate and Mg^{2+} are visible in strong density, with an average B-factor of $19 \text{ \AA}^2$. The γ -phosphate in both

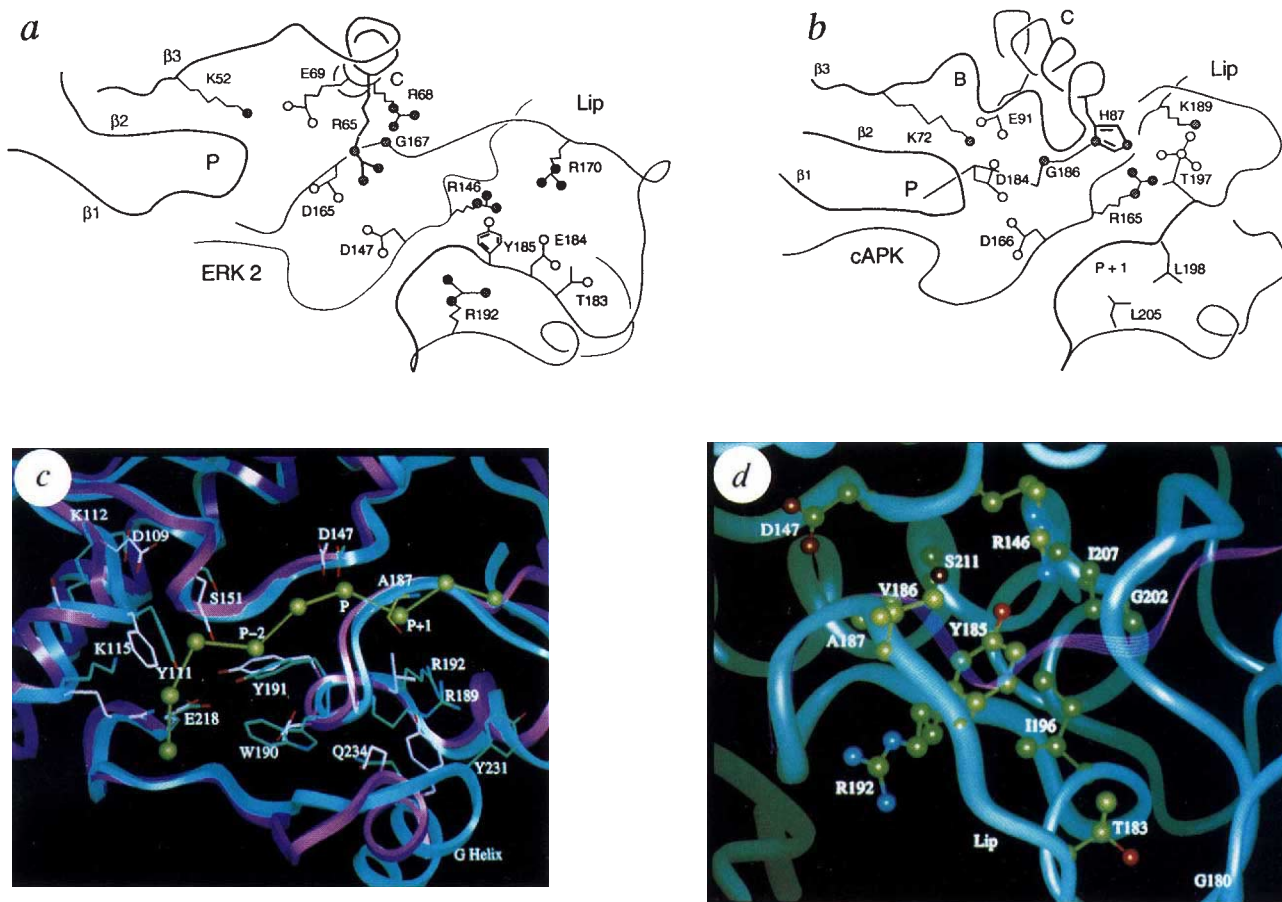


FIG. 4 *a*, Line drawing of the active site and phosphorylation lip in ERK2. *b*, Identical view of cAPK. Note the difference in separation between the phosphate anchor ribbon (P) and the phosphorylation lip (Lip), and the disposition of the catalytic residues Lys 52, Asp 147 and Asp 165. *c*, Superposition of ERK2 (cyan) and cAPK (magenta) of the specificity site region drawn in INSIGHT. The α positions of the PKI inhibitor are shown as green spheres. The side chains make contacts with PKI in cAPK²⁹. *d*, The phosphorylation sites of ERK2. Residues contacting Tyr 185 are Arg 146, Val 186, Ile 196, Gly 202, Ile 207 and Arg 192, and shown in CPK representation. The backbone trace of the phosphorylation lip of cAPK from residues 194 to 200 is shown as a transparent magenta ribbon to illustrate the position of the backbone of cAPK. *e*, Stereo diagram of the binding mode of Mg-ATP (the γ -phosphate of ATP is not shown). Side chains of residues in contact with Mg-ATP are shown.

structures is disordered, which suggests that a Michaelis-like complex has not been formed in either binary complex. The nucleotides are loosely wedged in the domain interface between Val 37 of the phosphate anchor ribbon and Leu 154 in strand $\beta 7$ (Fig. 4e), and make more contacts with the C-terminal domain than the N-terminal domain. Although few contacts are present, the Mg-ATP is deep in the back of the domain interface. The amino group (N6) of the adenine ring is hydrogen-bonded to the backbone carbonyl of Asp 104. The ribose O2' and O3' hydroxyls form a hydrogen-bonding network to Asp 109 (Glu 127) and Lys 112. In cAPK, Glu 127 and the arginine residue at P-3 of PKI form similar interactions with the ribose. As noted above, the binding to the ATP ribose ring by Lys 112 in ERK2, rather than by the P-3 residue in cAPK, may contribute to the specificity of ERK2 for hydrophobic residues at P-3. Lys 52 is not bound to any of the phosphates of ATP, and the close interaction between the conserved glycine residues in the phosphate-binding ribbon and the nucleotide observed in cAPK is absent in this crystal form of ERK2.

Discussion

The structural data presented here provide a wealth of information concerning the phosphoregulation and specificity of this kinase. First, the structure revealed that the phosphorylation site, Tyr 185, is buried, suggesting strongly that the phosphorylation lip must take up a completely different conformation when bound to its activator, MEK. The mechanism by which MEK is able to expose the tyrosine for phosphorylation is unclear. On the other hand, the ability of ERK2 to autophosphorylate Tyr 185 but not Thr 183²⁰ is readily apparent, from the positions of these two residues in the structure: Tyr 185 faces the active site, whereas Thr 183 faces away from the active site.

Second, comparison of ERK2 with inhibitor-bound cAPK suggests that the origin of the specificity of ERK2 and CDK2-related kinases for proline at the P+1 position is that Ala 187 (non-glycine) and Arg 192 of ERK2 fill and block the P+1 pocket observed in cAPK.

Third, comparison of the structure of this unphosphorylated ERK2 with cAPK suggests that both global and local conformational changes are involved in the activation of ERK2. Because of an apparent domain rotation, the catalytic-residues are misaligned, and as a result of the conformation of the phosphorylation lip, the substrate binding site is partially blocked. These mechanisms are a combination of those found in other phosphoregulated enzymes: phosphorylation acts sterically to block isocitrate binding⁴⁰ in isocitrate dehydrogenase; phosphorylation induces a domain rotation that forms the active enzyme in glycogen phosphorylase⁴¹.

Because both ERK2(Thr 183→Ala) and ERK2(Tyr 185→Phe) mutants are inactive²⁰, the phosphorylation of both residues is essential to form the active enzyme. The two phosphorylations are likely to promote domain closure and relieve steric constraints to substrate binding. In ERK2, Tyr 185 performs a steric role by blocking the formation of a Michaelis complex. Thr 183 may promote domain closure on phosphorylation by interacting with charged residues from the N-terminal domain such as Arg 65(His 87) and Arg 68(Arg 90) (Fig. 4a, b). Interestingly, the mutant ERK2(Thr 183→Glu) is partially active when phosphorylated by MEK on Tyr 185²⁰. Glutamate may replace the threonine PO₄ group in binding to the N-terminal domain. In cAPK, the Thr 197 phosphate forms ion pairs with residues that bridge the domain interface (Fig. 3d).

Domain motions are widely used in nature to regulate the activity of enzymes⁴². The domain separation in ERK2 appears to be modulated by the way in which the Asp-Phe-Gly sequence interdigitates with the side chain of the residue at position 65 of the C helix, as noted above. In addition to providing one of the catalytic residues, the Asp-Phe-Gly may also provide a switch that can support a closed, active conformation of the molecule or an open, inactive conformation, depending on the orientation

of the side chain at position 65 in the C helix (on one side or the other of Gly 167; Fig. 3d). The difference in conformation between ERK2 and cAPK is reminiscent of the conformational differences between the high- and low-affinity forms of haemoglobin⁴³, in which a change in interdigitation between residues in helix C in the α_1 chain and the FG corner (and extended β -strand) of the β_2 chain is the most prominent conformational difference.

The protein kinase family is fundamentally distinct from other known enzymes that catalyse phosphotransfer reactions. The ATP-binding site is in the centre of the molecule, whereas in enzymes such as hexokinase⁴⁴ the organic substrate is bound in the centre and the ATP binds towards the outside. This difference allows for the evolution of protein kinases with different specificities by alteration of surface structures. The great diversity in the observed topology of the phosphorylation lip (L₁₂; Fig. 4a, b) suggests that this lip structure is also a key design feature that can modulate both the specificity and mechanism of phosphoregulation.

Several questions are posed by the present study. The foremost of these is whether the structure of cAPK is a good model for the active conformation of ERK2 and other protein kinases. Second, what are the roles of the dual phosphorylation events in the activation of MAP kinases? Third, does the fact that Tyr 185 is buried in ERK2 explain the high degree of specificity of MEK? Questions such as these will be answered when the active and inactive conformations of the same protein kinase are solved and when the structures of other protein kinases such as MEK become available. □

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LETTERS TO NATURE

A convective model for the zonal jets in the atmospheres of Jupiter and Saturn

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THE atmospheres of Jupiter and Saturn are stably stratified in a region extending from the tropopause down to the level of the cloud tops; the underlying region is dominated by convective overturning^{1,2}. Horizontal velocities within the stable region decrease with altitude³, suggesting that they are driven by momentum transfer from the deeper flow. But the vertical structure within the convective region is still poorly understood¹. Its visible component at cloud level consists of alternating east-west zonal jets, with the more pronounced eastward jets attaining velocities in excess of 100 m s^{-1} and widths of about 10^4 km (refs 2 and 3). Here we propose a mechanism whereby the zonal jets result from convection cells resembling atmospheric Hadley cells on the Earth⁴. We demonstrate this mechanism using a rotating axisymmetric bowl of fluid, uniformly cooled at the free surface; radially overturning convection cells are established, the surface manifestation of which are pronounced azimuthal jets. A simple linear theory reproduces the main characteristics of the laboratory flow and predicts a relatively shallow penetration depth for the convection cells on Jupiter and Saturn.

Figure 1 shows the latitude dependence of the mean zonal wind on Jupiter. The velocity distribution outside the equatorial region can be reproduced by angular-momentum-conserving transport from lower to higher latitudes, followed by dissipation which reduces the angular momentum to its local planetary value². This suggests that the eastward jets may consist of a series of convecting cells, with dissipation occurring in the strong upwelling (or downwelling) zones at the outer edges of the cells.

Previous models of the jovian atmosphere have tended to focus on the stably stratified region, emphasizing the formation and long term survival of the Great Red Spot and other jovian eddies. Zonal jets have therefore tended to be produced by convenient controllable methods, rather than with realistic thermal forcing. For example, laboratory jets have been produced by heating and cooling at vertical boundaries^{5,6} and source-sink distributions⁷, whereas prescribed zonal jets have formed the bottom boundary condition for numerical simulations^{8–11}. Previous laboratory studies of thermally driven Hadley cells^{12–14} are more relevant to the convective region, but are strongly influenced by frictional boundary layers beneath solid upper boundaries.

Our laboratory model consisted of an insulated bowl mounted on a rotating table. This was filled with warm water and rotated anticlockwise. As the water temperature fell very slowly compared to the timescale of the convective motions, the dynamics

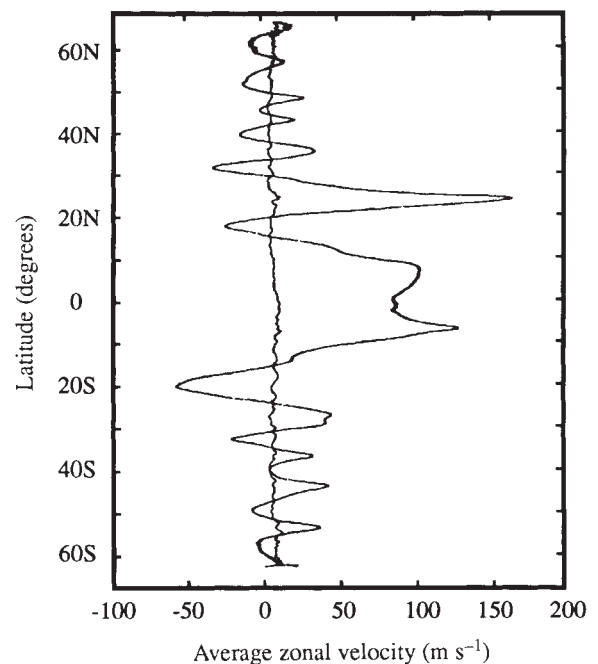


FIG. 1 Mean zonal winds and r.m.s. deviations on Jupiter calculated from cloud motions during the Voyager 1 Mission²².

could be considered to be quasi-steady. Uniform cooling from above resulted in the production of colder fluid in shallower regions where the volume per unit surface area was less. Because the radial temperature gradients were too large to be balanced by turbulent transport, convection cells developed to carry the radial heat flux. These were characterized by downwelling within intense cyclonic (anticlockwise) vortices balanced by more gradual upwelling in deeper regions (Fig. 2a). As relatively warm fluid neared the free surface and began to move radially outward towards the shallows, it accelerated anticyclonically (clockwise) so as to conserve angular momentum. This limited the radial dimension of the cell, enabling one or more azimuthal jets to form at the free surface (Fig. 2b).

If we assume that the specific angular momentum (defined by $fr^2/2 + ur$, where r is the radial coordinate, f is twice the angular velocity of rotation, $u(r, z)$ is the azimuthal velocity and z is the vertical coordinate) is conserved near the free surface of the cell⁴ extending radially from $r=r_1$ to $r=r_2$, then the surface velocity will be

$$u(r, 0) = \frac{fr_1}{2} \left(\frac{r_1}{r} - \frac{r}{r_1} \right) \quad (1)$$

Warm fluid surfaces at $r=r_1$, then accelerates anticyclonically (clockwise) until it sinks at $r=r_2$ where the maximum velocity is $-f\Delta r$ (to order $\Delta r/r_2$) where $\Delta r = r_2 - r_1$.

Downwelling in the convection cells involved rapid vertical accelerations which are difficult to analyse. But the jet regions are less thermodynamically active and can be described by the

thermal wind equation which governs most large-scale atmospheric motions¹⁵,

$$f \frac{\partial u}{\partial z} = -\frac{g}{\rho_0} \frac{\partial \rho}{\partial r} \quad (2)$$

where g is the gravitational acceleration, ρ is the local fluid density and ρ_0 is the mean density. Integrating equation (2) from the bottom ($z = -H$ where $H(r)$ is the fluid depth) to the free surface ($z = 0$) yields

$$u(r, 0) - u(r, -H) = -\frac{g\bar{H}}{f} \frac{\partial \sigma}{\partial r} \quad (3)$$

where $\bar{H}$ is the mean depth of the cell and σ is a dimensionless depth-averaged density defined by

$$\sigma(r) = \frac{1}{\rho_0 \bar{H}} \int_{-H}^0 \rho \, dz \quad (4)$$

where ρ_0 is the mean density of the cell (that is $\int_{r_1}^{r_2} \sigma \, dr = \Delta r$). Bottom friction in the laboratory experiments should ensure that $u(r, 0) \gg u(r, -H)$, so that the second term in equation (3) can be neglected. Integrating equation (3) from r_1 to r then yields an expression for the depth-averaged density,

$$\sigma(r) = \sigma(r_1) + \frac{f^2 r_1^2}{4g\bar{H}} \left(r^2/r_1^2 - 2 \ln \left(\frac{r}{r_1} \right) - 1 \right) \quad (5)$$

The density increases with r until sinking occurs at a critical density $\sigma(r_2)$. Calculating $\sigma(r_2)$ from equation (5) and expanding the logarithmic term as a power series¹⁶ yields a cell width (to order $\Delta r^2/r_1^2$) of

$$\Delta r = 2^{1/2} R \quad (6)$$

where $R = (g\Delta\sigma\bar{H})^{1/2}/f$ is an internal deformation radius based on the depth-averaged horizontal density change, $\Delta\sigma = \sigma(r_2) - \sigma(r_1)$, and the average depth of the cell. By equating the heat flux through the air and water thermal boundary layers it can also be shown that $\Delta\sigma \approx \lambda\alpha\Delta T$, where α is the coefficient of thermal expansion for water, ΔT is the difference between the mean temperature of the fluid and the air above, and λ depends only on the physical properties of air and water¹⁶ (that is, thermal conductivity and diffusivity, thermal expansion coefficient and kinematic viscosity). For the range of parameters used here $\lambda = 0.03$, which is consistent with measured values of $\Delta\sigma = (0.05 \pm 0.02)\alpha\Delta T$ taken from horizontal temperature profiles.

Photographs (Fig. 2b) and video recordings from 25 separate experiments were used to measure the radial width and maximum velocity of the jets over a wide range of rotation rates and temperature differences. An example of the zonal velocity distribution is shown in Fig. 3. For flows in which R was significantly less than the bowl radius, the mean jet width was found to be $(5.7 \pm 0.6)R$. Maximum jet velocities from the same experiments were $(0.92 \pm 0.12)fR$. These results are consistent with the theoretical scaling, although the laboratory jets tend to be wider and slower than predicted by the linear theory. This can be attributed to the nonlinear momentum fluxes associated with large-amplitude meanders¹⁷, which tend to break the angular momentum constraint. Measured wavenumbers of these disturbances (over the same parameter range) scaled closely with R^{-1} , suggesting they were produced by baroclinic instability¹⁵.

Many of the dynamical characteristics of the flows in our model seem to be relevant to the convective jets in the jovian and saturnian atmospheres below cloud-top level. The jets are qualitatively similar in appearance, and the basic assumptions of thermal wind balance and angular momentum conservation, which apply to the experiments, are also appropriate for planetary atmospheres. The major uncertainty in applying the model quantitatively relates to the penetration depth of the convection cells on Jupiter and Saturn. Large depths, comparable to the jet widths ($\sim 10^4$ km), would result in dynamical regime based on

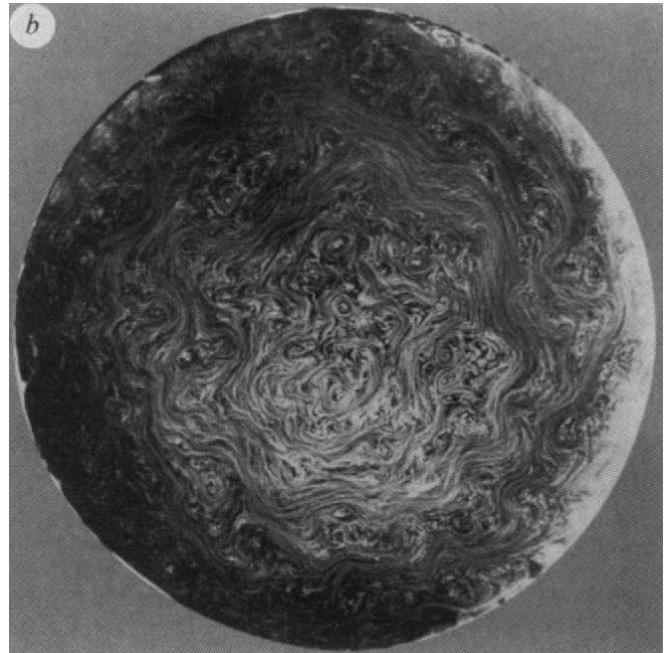
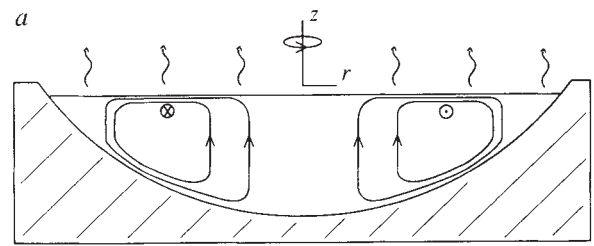


FIG. 2 a, Schematic side view of an experiment with a single convection cell cutting the plane of the page at two azimuthal locations. The surface flow is into the page on the left and out of the page on the right. b, An example of the surface flow in a rotating bowl cooled from above and insulated at the sides. The flow is visualized by a streak photograph of floating aluminium powder. A number of meandering jets travel anticyclonically (clockwise) around the bowl. They are separated by rings of cyclonic (anti clockwise) vortices which form the downwelling arm of the convection cells by carrying cooled surface water in spiralling motions to the bottom. These structures are closely related to those observed in rotating convection experiments over a flat bottom²³⁻²⁵. Without topography, downwelling in cyclonic vortices would be surrounded by anticyclonic upwelling motions. But, with topography, broad areas of slow upwelling develop in deeper regions, resulting in the coherent jet motions. The bowl shown here was 46 cm in diameter at the free surface and the maximum fluid depth was 11 cm. The rotation rate correspond to $f = 1.4 \text{ s}^{-1}$ and the temperature difference between the air and water was $\Delta T = 12^\circ \text{C}$. Other runs covered the range $f = 0.5 - 5.0 \text{ s}^{-1}$ and $\Delta T = 10 - 32^\circ \text{C}$. The lowest rotation rate corresponded to flows in which the predicted jet width slightly exceeded the bowl radius.

observable quantities are obtained when shallower depths of a few scale heights² (~ 100 km), are assumed. For example, using a value of $\Delta\sigma\bar{H} = 1.2 \text{ km}$ for Jupiter²¹ yields a cell width of order $R \sim 10^4 \text{ km}$ and a maximum jet velocity of order $fR \sim 100 \text{ m s}^{-1}$, as suggested by observations. Uncertainties in the convective penetration depth should be resolved by *in situ* observations by the Galileo descent probe scheduled for late 1995.

One important difference between the laboratory and planetary flows is the presence of large amplitude meanders along the laboratory jets (Fig. 2b). The vorticity gradient associated with the curvature of the planets strongly limits meridional motions.

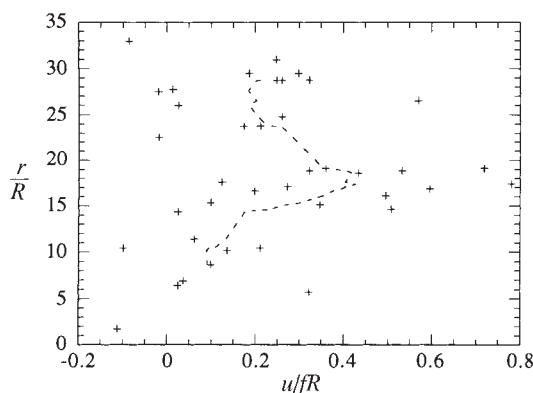


FIG. 3 An example of the distribution of zonal velocities in the laboratory flow. The zonal velocity u is rendered non-dimensional by dividing by the product of the rotation rate f and the deformation radius R on the x axis, while the radial coordinate r is rendered non-dimensional by dividing by R on the y axis. The crosses represent instantaneous readings, whereas the dashed curve is the zonal average based on the five closest points to a given radius. All readings were taken within a single time interval of three rotation periods, so that the large spread is almost exclusively associated with meanders in the jet path. Despite this, at least one strong jet near mid-radius is easily identified. The counter-rotating (anticlockwise) motions are comparatively very weak and are completely eliminated by zonal averaging. The bowl in this example was 34 cm in diameter at the free surface and the maximum fluid depth was 7.4 cm. The rotation rate corresponded to $f = 2.0 \text{ s}^{-1}$ and the temperature difference between the air and water was 1.3°C .

columnar convection.^{18,19,20} However, realistic estimates of This both stabilizes the jets and aligns co-rotating eddies along latitude circles. Under these conditions the westward component of the eddies can contribute significantly to the zonally averaged velocity field at specific latitudes. For instance, the largest westward peak in the zonally averaged velocity profile of Jupiter (Fig. 1) corresponds to the latitude of the westward half of the Great Red Spot²². We would therefore expect the zonally averaged westward velocities on the planets to be far more significant than those in the laboratory (Fig. 3). It should be emphasized, however, that this discrepancy is magnified by the use of zonal averages and does not represent any fundamental differences in the convective dynamics of the two systems. $\square$

X-ray emission from the radio hotspots of Cygnus A

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THE giant elliptical galaxy Cygnus A is regarded as the archetypal high-luminosity radio galaxy. These objects contain radio jets: an unknown energy source in the galactic nucleus generates two collimated outflows in opposite directions, which create radio hotspots where they interact with the surrounding intergalactic medium. Many radio galaxies are known to emit X-rays; in some cases these have been shown to come from the central core region, and in others from an extended galactic halo. Here we report the detection of X-ray emission from the hotspots of Cygnus A, obtained with the High Resolution Imager of the Rosat satellite. We show that an explanation based on thermal bremsstrahlung from a hot gas is inconsistent with radio polarization data and that a synchrotron model would require a population of relativistic electrons distinct from that responsible for the radio emission. Inverse Compton scattering of radio photons by relativistic electrons in the hotspots is, however, able to account for the observed X-ray intensity. A model based on this latter mechanism allows us to calculate the average magnetic field strength and the energy content of the relativistic electrons in the hotspots.

The X-ray image of Cygnus A is shown in Fig. 1. The dominant X-ray feature is emission from hot diffuse gas; the Cygnus A galaxy is at the centre of a poor cluster of galaxies^{1,2}. Although this thermal emission is roughly azimuthally symmetric around the galaxy, there are clear signatures of hydrodynamic interaction between the radio source and the intracluster medium (ICM); (C.L.C. *et al.*, manuscript in preparation). A further departure from circular symmetry is the excess X-ray emission from the radio hotspots at the ends of the lobes. The peaks of the X-ray hotspots are separated by $122.4'' \pm 2''$, while the radio hotspots are separated by $123.4'' \pm 2''$. The western X-ray hotspot is small, with a deconvolved full-width half-maximum (FWHM) $\leq 4''$, roughly comparable to the radio dimensions. The eastern hotspot is extended, with an X-ray structure which follows closely that seen in the radio. The X-ray emission coincides both with the radio hotspot, and with a 'ridge' of bright radio emission along the northern edge of the eastern lobe. The remarkable similarity of the separations, together with the agreement in morphology, convinces us that the X-ray emission is co-spatial with the radio emission.

Flux estimates of the X-ray hotspots were made by summing the counts from each hotspot area and using the surface brightness of the diffuse gas at the same distance from the centre (but at other position angles) for the background. We find 75 ± 15 (west) and 113 ± 18 (east) net counts. These numbers correspond to luminosities (0.5–2 keV) of 0.9 (west) and 1.3 (east) $\times 10^{42} \text{ erg s}^{-1}$. Flux densities are plotted together with the radio data and optical upper limits in Fig. 2.

The hypothesis that the X-ray emission from the hotspots comes from a thermal gas does not seem to be supported by the available data. For a uniform plasma in a spherical volume with a radius of $2.3''$ (the approximate size of the point response function) and a temperature of 1 keV, a density of 0.2 electrons cm^{-3} would be required to explain the observed X-ray emission. Even larger densities would be necessary for smaller volumes, or temperatures significantly different from 1 keV. But from the lack of frequency dependence of the fractional polarization at fixed spatial resolution, Dreher, Carilli and Perley³ have derived

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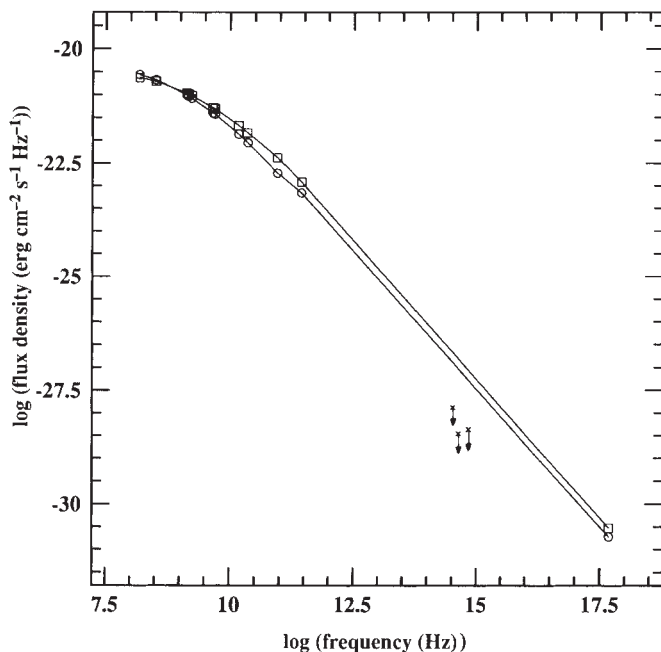


FIG. 2 Spectra of the hotspots. The eastern hotspot, D, is indicated by boxes and values for the western hotspot, A, are shown by circles. The radio data are taken from Carilli *et al.*⁸. The optical upper limits (crosses) are 5σ values from H. J. Röser (personal communication), and assume a visual extinction of $1.26 \text{ mag}^{1,11,12}$. The 2 keV flux densities were calculated for a spectral distribution defined by absorption of a column density of hydrogen, $N_H = 3.3 \times 10^{21} \text{ cm}^{-2}$ (the galactic value for this direction) and a power law of index $\alpha = 0.75$. The corresponding 0.5–2 keV fluxes are 6.6 (western hotspot) and 9.8 (eastern hotspot) $\times 10^{-14} \text{ erg cm}^{-2} \text{ s}^{-1}$. For $\alpha = 0.5$, the fluxes should be increased by 0.2×10^{-14} , and for $\alpha = 1$, they decrease by the same number.

upper limits to the internal thermal gas density of $\sim 4 \times 10^{-4} \text{ electrons cm}^{-3}$. Therefore, if the X-ray emission from the hotspots were to be thermal, the gas responsible would have to be external to the radio volume. Dense clumps of compressed gas formed from the ICM cannot fail to be magnetized, and if they were in front of the radio hotspots, one would expect a significant increase in rotation measure and/or depolarization over the hotspots relative to the rest of the radio source. No such increase is seen³. Moreover, such a clump of gas cannot be made from the smooth ICM because even a strong shock cannot compress the ICM higher than $\sim 0.05 \text{ electrons cm}^{-3}$ (from an ambient density of $0.012 \text{ electrons cm}^{-3}$ at $64''$ from the centre). Thus we find that the only allowed thermal model—pre-existing high-density regions lying behind each hotspot when viewed from the Earth—is too contrived to be realistic.

The spectral index, α (flux density $\propto \nu^{-\alpha}$) between the radio and X-ray is 1.2 . However, the optical upper limits (Fig. 2) are a factor of at least 40 below the value expected from such a spectrum. Hence the X-rays cannot be explained by a simple extrapolation of the radio power-law to high energies. If the X-ray emission is synchrotron radiation, we must postulate a population of ultra-high-energy relativistic electrons which is co-spatial with the lower-energy electrons, but is generated by some process other than the shocks responsible for the radio-emitting population. This statement is based on the premise that shocks produce monotonically decreasing power laws, and the observation that the optical limits imply a cutoff in the electron spectrum which is well below the energies required for X-ray emission. Although such a 'double-peaked' energy distribution appears somewhat *ad hoc*, there may be a mechanism that could generate the high energy component: the 'proton induced cascade' (PIC). Mannheim, Krulls and Biermann⁴ have argued that the shocks

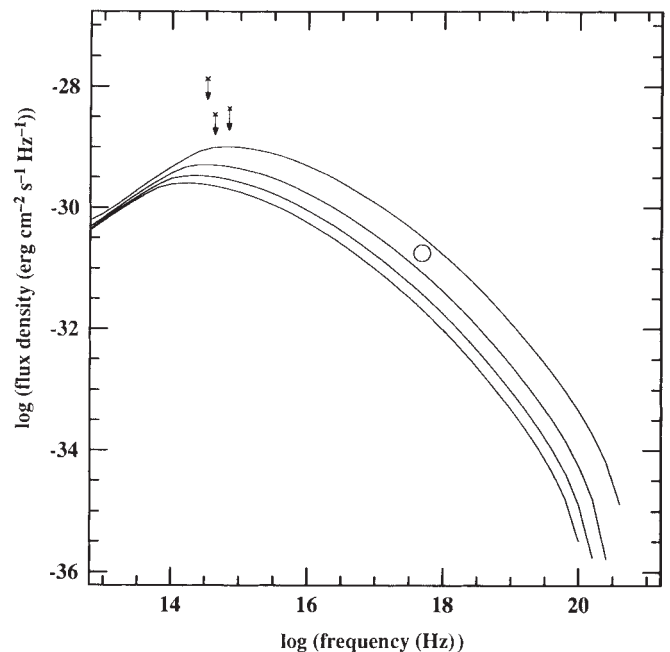


FIG. 3 Predicted synchrotron self-Compton (SSC) spectra (solid lines) for the western hotspot, A. Photon energy densities were derived from the high-resolution radio maps of Carilli *et al.*¹⁰. We estimate the dimensions of the emitting volumes as cylinders with a radius of $0.83''$ ($0.6''$) and a length of $2.5''$ ($1.8''$) for the western (eastern) hotspot. A broken power law was used for both the observed radio photons (327 MHz to 375 GHz) and for the corresponding population of relativistic electrons. These 'input' spectra are defined by spectral indexes of 0.5 (below ν_{break}) and 1.0 (above ν_{break}), with the break occurring at 5.5 GHz (west) and 14 GHz (east). Flux densities at 1.4 GHz are $S(\text{east}) = 118.9 \text{ Jy}$, and $S(\text{west}) = 108.3 \text{ Jy}$. Values of the magnetic field strength (required for the amplitude of the electron spectra and hence the scattered radiation spectra) are $100, 200, 300$ and $400 \mu\text{G}$ (for the four simulated spectra, top to bottom). The X-ray flux density (open circle) and optical upper limits (crosses) are the same as those plotted in Fig. 2. The size of the circle used for the X-ray flux density is $\sim \pm 40\%$, which is roughly twice the statistical uncertainty in the net counts.

in radio hotspots will produce a population of relativistic protons in addition to the electrons responsible for the radio synchrotron emission. These will initiate a cascade with eventual deposition of energy in X- and γ -ray emission; that is, the observed PIC spectrum arises from synchrotron emission from a population of electrons which is continuously replenished by the cascade from higher energies.

If the PIC mechanism operates in the Cygnus A hotspots with an intensity sufficient to generate the observed X-ray emission, a number of conditions must be met. First, a supply of protons is required, which would imply that the jets consist of electrons and protons rather than electrons and positrons. Second, the shock acceleration must provide a proton distribution extending to an energy of at least $\gamma_p \approx 10^{11}$ in order to initiate the cascade. Third, the average magnetic field strength must be significantly greater than $300 \mu\text{G}$ in order that the gyro-radius of the protons does not exceed the hotspot size. All of these conditions are possible in the hotspots, but none is yet required by observational constraints.

Another way to explain the X-ray emission is as synchrotron self-Compton (SSC) emission; that is, the result of inverse Compton scattering of synchrotron-produced photons by the same population of relativistic electrons that initially generated the photons. From the observed radio spectrum and emitting volume we are able to calculate the synchrotron photon energy density, and (for trial values of the magnetic field) the energy distribution of the relativistic electrons responsible for the radio emission⁵. Thus we are able to predict inverse Compton spectra

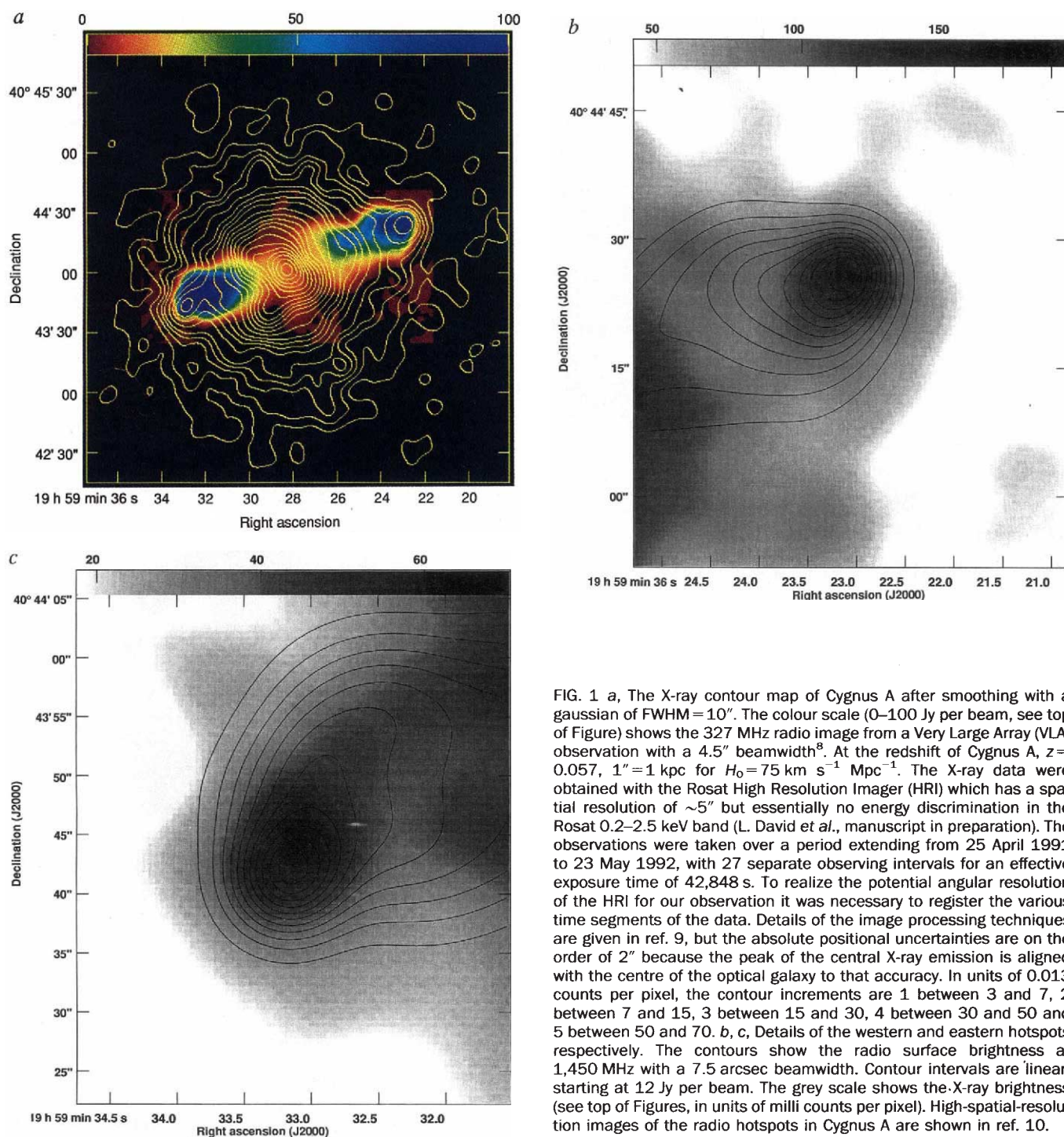


FIG. 1 *a*, The X-ray contour map of Cygnus A after smoothing with a gaussian of FWHM = 10". The colour scale (0–100 Jy per beam, see top of Figure) shows the 327 MHz radio image from a Very Large Array (VLA) observation with a 4.5" beamwidth⁸. At the redshift of Cygnus A, $z = 0.057$, $1'' = 1$ kpc for $H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The X-ray data were obtained with the Rosat High Resolution Imager (HRI) which has a spatial resolution of $\sim 5''$ but essentially no energy discrimination in the Rosat 0.2–2.5 keV band (L. David *et al.*, manuscript in preparation). The observations were taken over a period extending from 25 April 1991 to 23 May 1992, with 27 separate observing intervals for an effective exposure time of 42,848 s. To realize the potential angular resolution of the HRI for our observation it was necessary to register the various time segments of the data. Details of the image processing techniques are given in ref. 9, but the absolute positional uncertainties are on the order of 2" because the peak of the central X-ray emission is aligned with the centre of the optical galaxy to that accuracy. In units of 0.013 counts per pixel, the contour increments are 1 between 3 and 7, 2 between 7 and 15, 3 between 15 and 30, 4 between 30 and 50 and 5 between 50 and 70. *b*, *c*, Details of the western and eastern hotspots respectively. The contours show the radio surface brightness at 1,450 MHz with a 7.5 arcsec beamwidth. Contour intervals are linear, starting at 12 Jy per beam. The grey scale shows the X-ray brightness (see top of Figures, in units of milli counts per pixel). High-spatial-resolution images of the radio hotspots in Cygnus A are shown in ref. 10.

with the average magnetic field strength as the single variable. This model involves the simplifying assumption that the field strength and particle spectra are spatially uniform throughout the emitting volumes which we approximate as cylinders in each case. We used the IC computer code of Band and Grindlay^{6,7} (supplied by J. Webb) to produce the SSC spectra shown in Fig. 3.

The values of magnetic field strength that predict an SSC spectrum in agreement with our observed flux densities are $158 \pm 17 \mu\text{G}$ (western hotspot) and $246 \pm 22 \mu\text{G}$ (eastern hotspot). The quoted errors are calculated from the statistical uncertainties in the primary measurements of net counts and do not include contributions from systematic effects. Varying the emitting volume by 50% changes the resulting field strength by <20%.

The standard method used to estimate magnetic field strengths in extragalactic radio sources is to assume a minimum energy configuration for the field and particles, that is, the summed energy density in fields and relativistic particles is a minimum⁵. This corresponds roughly to equipartition in energy density between fields and particles. The validity of this minimization assumption has never been tested, but for the volumes and spectra used to calculate the SSC models for the hotspots of Cygnus A, magnetic field strengths corresponding to minimum energy and minimum non-thermal pressure bracket (and are within 20% of) those from the SSC calculations. Note that this result depends on the assumed ratio (K) of energy densities in relativistic protons to that in electrons. We have assumed $K = 0$, but if protons were to contribute most of the particle energy

density, $u(\text{part})$, (for example if $K \approx 100$), then either the hot-spots are far from equipartition ($u(\text{part}) \gg u(\text{field})$) or the field is much greater than calculated above and the observed X-rays come from direct synchrotron emission rather than inverse-Compton emission. If part of the observed X-ray flux were to come from any process other than SSC emission, then the column density of relativistic electrons would be less than that of our SSC model and a larger average field strength would be required to produce the observed radio emission. Therefore, regardless of the actual emission process of the observed X-rays,

the field strengths derived above are strict lower limits to the average fields in the hotspots because the SSC process is a necessary consequence of synchrotron emission (that is, the presence of relativistic electrons and target photons). In spite of the uncertainties in emitting volumes, and the simplifying assumption that the emission region is uniform in particle density and magnetic field strength, we consider the agreement between the SSC-derived magnetic field and that calculated from the standard minimization assumptions to be convincing evidence that the X-ray emission is, in fact, SSC emission. $\square$

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Metallic behaviour in a Pt_{309} cluster revealed by ^{197}Au Mössbauer spectroscopy

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SMALL metal clusters, containing of the order of 10 to 10,000 atoms, are of interest both for exploring the fundamental question of how atomic-scale properties develop into bulk properties and because such clusters are expected to have interesting optical, electronic and magnetic properties¹. The expectation is that bulk-like metallic behaviour will become increasingly evident as the cluster size increases, but at what stage bulk properties appear is not yet clear. Here we present results of a study of the platinum cluster compound $\text{Pt}_{309}(\text{Phen}^*)_{36}\text{O}_{30 \pm 10}$ (refs 2–4), in which we use Mössbauer spectroscopy to investigate the bonding environment of the Pt atoms. Because Pt lacks a good Mössbauer isotope, we transform a fraction of the ^{196}Pt atoms in the clusters into Mössbauer nuclei ^{197}Au by neutron irradiation. The resulting spectra show that the inner (147-atom) core of the Pt cluster exhibits a metallic character like that in the bulk metal, whereas the surface atoms are not bulk-like. These bulk-like metallic properties may be acquired by metal-cluster cores as small as about 150 atoms.

It has been shown^{2–4} that chemical synthetic methods can be used to prepare metal-cluster compounds with well defined stoichiometry, in which polyhedral metal cores containing up to several hundreds of atoms are surrounded by ligand shells. Because the size and structure of all of these clusters are identical, they provide ideal model systems for studying metal-cluster behaviour². The Pt_{309} cluster (Fig. 1) is the four-shell member of a series of n -shell magic-atom-number metal clusters M_{13} , M_{55} , M_{147} , M_{309} , M_{561} , ... , which are obtained by surrounding a given metal atom (M) progressively with shells of additional atoms of its kind. The two-shell M_{55} core is found in a series of cluster molecules synthesized by Schmid and co-workers^{3,4}. In addition, the four-shell $\text{Pt}_{309}(\text{Phen}^*)_{36}\text{O}_{30 \pm 10}$ and the five-shell $\text{Pd}_{561}(\text{Phen}^*)_{195 \pm 5}$ compounds (here Phen is phenanthroline and

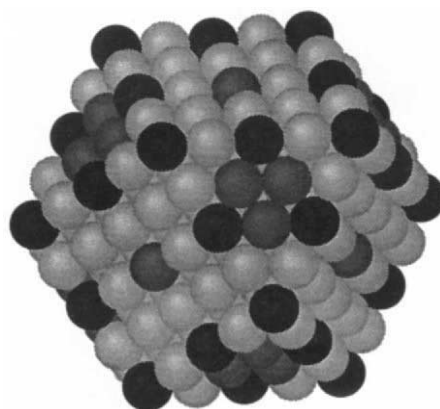


FIG. 1 The cuboctahedral Pt_{309} core of $\text{Pt}_{309}(\text{Phen}^*)_{36}\text{O}_{30 \pm 10}$. The 36 Pt atoms ligated by Phen^* at the 12 vertices and the 24 midpoints of the ribs are indicated by black spheres; we have assumed 14 O_2 molecules to be bonded to the Pt atoms at the centres of the flat faces, and these are indicated by dark-grey spheres. Note that on the triangular faces, three atoms would share the O_2 molecule. The light-grey spheres indicate the 96 unligated atoms.

Phen^* is a phenanthroline derivative) have been synthesized^{3,4} and investigated by physical measurements^{2,5–9}. The two-, four-, and five-shell compounds have the cuboctahedral (face-centred cubic) arrangement of the metal atoms (as in the bulk metal). No three-shell compound has yet been found. As a general conclusion of these studies, the 'surface' metal atoms in the outer shell of the metal core do not show 'metallic' behaviour, both because of charge transfer between these atoms and the surrounding ligand molecules, and because of the reduction in coordination number of these surface atoms. The remaining inner-core metal atoms show increasing tendency for 'metallic' behaviour as n increases. (By 'metallic' we mean properties analogous to those found for the corresponding bulk metal.)

In view of the interesting results obtained by previous ^{197}Au Mössbauer spectroscopy studies on the Au_{55} cluster compounds^{5–7}, extension of this technique to larger clusters appeared very desirable. Lacking Mössbauer-active Pt or Pd isotopes, we have developed a method to study Pt cluster compounds by ^{197}Au Mössbauer spectroscopy. By irradiating a Pt

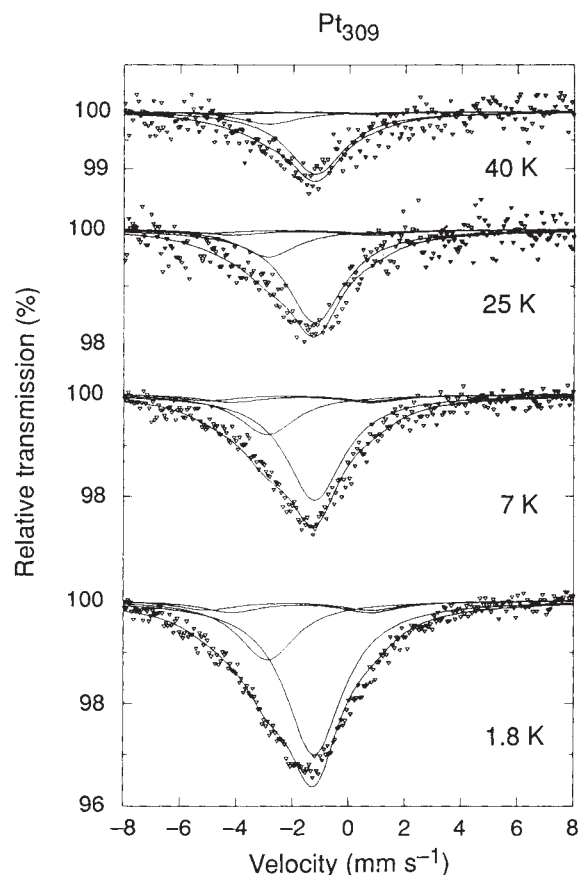


FIG. 2 Representative Mössbauer spectra of $\text{Pt}_{309}\text{Phen}^*_{36}\text{O}_{30 \pm 1.0}$ taken at 40, 25, 7 and 1.8 K. The least-squares fits of the spectra (full line, running through the data points) were calculated using the transmission integral method for the absorber and restricting the linewidths of source and absorber to the natural linewidth of 0.923 mm s^{-1} . The Lorentzian simulations of the spectra corresponding to the individual 'sites' are meant only as guides to the eye.

cluster sample with thermal neutrons, a fraction of the Pt atoms is transformed into ^{197}Au by the nuclear reaction $^{196}\text{Pt} + n \rightarrow ^{197}\text{Pt}$, followed by $^{197}\text{Pt} \rightarrow ^{197}\text{Au} + e^- + \bar{\nu}_e + 0.6 \text{ MeV}$ (the half-life of ^{197}Pt is 18 h) (e^- and $\bar{\nu}_e$ denote an electron and an antineutrino). The ^{197}Au nucleus thus formed is in an excited state and decays with a half-life of 1.892 ns, emitting a (Mössbauer) photon of energy 77 keV. In this way, an irradiated Pt cluster sample can be the source in ^{197}Au Mössbauer experiments in transmission geometry, using a very thin ($37 \mu\text{m}$) gold

foil as absorber. A similar method has previously been applied to CoPt alloys¹⁰. It would be profitable to extend it to other Pt compounds or catalysts.

From the neutron cross-sections, irradiation time (24 h) and neutron flux ($2 \times 10^{13} \text{ cm}^{-2} \text{ s}^{-1}$), we estimate a chance of $<10^{-6}$ for a Pt_{309} cluster to capture a neutron. The chance for capture of two neutrons is thus negligible, so we measure effectively on AuPt_{308} clusters, with Au randomly replacing a Pt atom. In view of the high resistivity of the $\text{Pt}_{309}\text{Phen}^*_{36}\text{O}_{30}$ compound at low temperatures ($>10^6 \Omega \text{ cm}$)¹¹, the electron, emerging with high kinetic energy from the $^{197}\text{Pt} \rightarrow ^{197}\text{Au}$ reaction, will not be replaced fast enough to neutralize the cluster within the characteristic time (10^{-9} s) of Mössbauer spectroscopy. On the other hand, the electronic relaxation processes within the cluster will be short with respect to this time, so that the electronic configuration within the (ionized) AuPt_{308}^+ cluster will have stabilized.

Mössbauer spectra were taken at seven temperatures up to 60 K (Fig. 2). The fast decrease of absorption intensity with temperature, at temperatures far below the normal Debye temperature Θ_D (that is, much faster than for the bulk), is an unmistakable signature of a cluster, as it arises from the centre-of-mass motion of the entire cluster¹². These so-called intercluster vibrations impose an additional Debye–Waller factor (f -factor, recoil-free fraction) upon the f -factors associated with the intracenter vibrations of the atoms within the cluster. The structure of the cluster molecule is not known exactly, because single crystals are not available. But powder X-ray diffraction data⁴ confirm the f.c.c. packing, with interatomic distances equal to those in bulk Pt (within errors). Observed widths of the X-ray reflections agree well with the expected size of the Pt_{309} core, and high-resolution electron microscopy confirms⁴ both cluster size and f.c.c. packing.

The 147 inner-core atoms all have 12 metal neighbours, as in the bulk metal, and are taken together as one site. Due to their cubic symmetry, they will exhibit no quadrupole splitting (QS). Chemical analysis indicates that 36 Phen^* ligands and 10–20 O_2 ligands are present. On the basis of symmetry and geometrical considerations, we assume these ligands to be bonded to Pt atoms as shown in Fig. 1, leaving most of the surface unligated. The definition of the surface sites (Phen^* , O_2 , unligated), as used in our analysis of the Mössbauer spectra is given in Fig. 1 and Table 1.

The relative contributions to the Mössbauer spectrum of the four metal sites defined above, as well as the total absorption intensity, can be calculated on the basis of the site abundances just discussed (Fig. 1), together with the Debye–Waller factors (f -factors) associated with the intracenter vibrations of the individual sites (f_{intra}), and with the intercluster vibration of the cluster as a whole (f_{inter}). As described previously⁵, f_{inter} is determined within a Debye model, using the known cluster mass, and with the Debye temperature Θ_D associated with these intercluster vibrations as adjustable parameter. This Θ_D is easily extracted from the temperature dependence of the seven spectra measured. The f_{intra} are determined by the intracenter vibrational modes, which are calculated with a molecular dynamics method, using the known atomic masses and the spring-constant C describing the Pt–Pt bond-strength as an adjustable parameter. They depend on the local site-symmetry and coordination. From our present fits we obtain $C \approx 26 \pm 5 \text{ N m}^{-1}$ and $\Theta_D \approx 17 \pm 3 \text{ K}$, comparing favourably with $C \approx 20 \text{ N m}^{-1}$, $\Theta_D \approx 15 \text{ K}$ as obtained earlier from low-temperature specific heat data⁹.

The independently refined Mössbauer parameters from our analysis are, besides the intensities mentioned above, the QS and the isomer shifts (IS). In the present case, the QS-parameter arises from non-spherical charge distribution of the 5d and 6p valence electrons of the Au atom. The IS is determined mainly by the 6s electron charge density at the Au nucleus, and is therefore crucial in deciding whether the Au environment is like, or unlike, that in the metal.

TABLE 1 Mössbauer spectroscopy parameters for Pt_{309}

Site	IS (mm s^{-1})	QS (mm s^{-1})	N	I (%)
Core	0.00 ± 0.05	0.0 ± 0.05	1	62
Unligated	1.7 ± 0.1	0.0 ± 0.1	3	23
Phen^*	0.9 ± 0.4	5.7 ± 0.4	2	7
O_2	0.5 ± 0.4	5.0 ± 0.4	2	8

Values for the Mössbauer parameters IS (isomer shift) and QS (quadrupole splitting), obtained for the various sites in the Pt_{309} cluster. The IS follows from the centre of gravity of a subspectrum, the QS from the peak-separation in case of a split subspectrum. Note that the IS values are with respect to that of the bulk metal. N denotes the number of atom positions of different local symmetry combined into a site, I denotes the estimated contribution to the total intensity at $T = 1.8 \text{ K}$. The large I for the core sites ensures unambiguous determination of its IS and QS values. For the Phen^* and O_2 sites the values are relatively uncertain due to their small abundances.

The QS- and IS-parameters obtained are given in Table 1. The parameter values depend on such factors as the local symmetry, the type of ligand, and so on; we note that the QS is zero for bulk metal, in view of the cubic symmetry. Furthermore, the IS values are given with respect to that for ^{197}Au in bulk Pt metal (-1.22 mm s^{-1}), earlier determined for Au foil relative to a $^{197}\text{Au}:\text{Pt}$ (bulk) source¹³.

The most striking feature in Table 1 is that the IS of the inner-core sites in $^{197}\text{AuPt}_{308}^+$ equals, within the experimental error of $\pm 0.05 \text{ mm s}^{-1}$, that found for ^{197}Au in bulk Pt. As the interatomic distance for this cluster is also equal to the Pt bulk value, this warrants the conclusion that the 147 atoms of the inner core behave as a minute piece of bulk Pt (the IS depends sensitively on interatomic distance). For the 13-atom inner core of the Au_{55} clusters, we have found (in earlier studies) small but significant deviation of the IS from the bulk value. The interatomic distance in Au_{55} is also $\sim 3\%$ smaller than in the bulk^{6,7}.

The implication seems to be that for $\text{Pt}_{309}\text{Phen}_{36}\text{O}_{30 \pm 10}$ the effects of charge transfer between ligands and metal atoms, as seen by Mössbauer spectroscopy, are restricted to the first layer of surface atoms. This is perhaps not unexpected as far as the Au 5d electron density is concerned, as the d-orbitals are known to have strongly localized character. The IS value, however, probes mainly the density of the more itinerant 6s electrons, and it is therefore interesting in itself that for the inner atoms no measurable effect of the surface-ligation is seen. We note that the 'metallic' behaviour for the inner-core atoms in $\text{Pt}_{309}\text{Phen}_{36}\text{O}_{30 \pm 10}$ has also been inferred from the low-temperature specific heat⁹, as well as from ^{195}Pt -NMR measurements⁸. A discussion of these results falls outside the scope of this Letter.

Lastly we comment on the parameter values found for the other sites. The values of IS and QS for atoms at sites coordinated by Phen^* and O_2 are in a region common for Au surface atoms in clusters⁷, and for various linearly coordinated, nominally Au(I) compounds, as might be expected. It is surprising that

the unligated surface sites exhibit a QS value of zero, with an IS corresponding to nominally Au(V) compounds. On the other hand, similar values have been found for (central) Au atoms in small Au clusters, such as Au_8 , Au_9 and Au_{11} (see, for example, ref. 6 and references therein). None the less, we have at present no explanation for this result.

Further refinement and examination of alternative models for the location of O_2 molecules (and thus of part of the bare surface sites) is in progress. But the IS obtained for the core sites will not be substantially influenced by alternative choices for the different surface sites, nor by improvement of statistics and by further refinement, and our conclusion that the core sites experience an s-electron density comparable to that observed for an Au atom in bulk Pt metal should not be sensitive to these refinements. $\square$

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Gas-phase self-assembly of endohedral metallofullerenes

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METALLOFULLERENES^{1,2} consist of metal atoms trapped inside closed fullerene cages. Virtually nothing is known about the mechanism of metallofullerene synthesis, although the possibility of a condensed-phase mechanism has been suggested in recent studies^{3,4}. Here we show that laser vaporization of a La_2O_3 /graphite rod produces a number of LaC_{60}^+ isomers, including the endohedral metallofullerene and a variety of different isomers in which lanthanum seems to be bound to polycyclic polyyne rings. When heated, nearly all of the different ring isomers convert spontaneously into metallofullerenes, trapping the metal atom inside the fullerene cage with remarkably high efficiency ($>98\%$). We suggest that in the first step of this annealing process the lanthanum atom acts as a nucleation centre and the carbon rings arrange themselves around the lanthanum atom before converting into a fullerene cage.

Recent studies have shown that fullerenes can be synthesized by annealing polycyclic polyyne rings^{5,7}. The work described here was undertaken to determine whether a similar mechanism could result in the formation of metallofullerenes. The experimental approach has been described previously^{6,8}. LaC_{60}^+ ions were generated by laser vaporization of a La_2O_3 /graphite com-

posite rod¹, and selected with a quadrupole mass spectrometer. Pulses (50 μs) of LaC_{60}^+ ions were injected (at various kinetic energies) into a drift tube containing ~ 5 torr of He. The ions travel across the drift tube under the influence of a weak electric field, and the drift time depends on the geometry of the cluster⁹. Clusters with compact geometries have shorter drift times than clusters with less compact geometries.

Figure 1 shows the drift-time distributions recorded for C_{60}^+ and LaC_{60}^+ using low injection energies. These distributions essentially reflect the isomer populations generated by the source. The distribution for C_{60}^+ has been discussed in detail previously⁶. The narrow peak at $\sim 800 \mu\text{s}$ is due to the fullerene, and the broad distribution centred at $\sim 1,600 \mu\text{s}$ has been assigned to a variety of polycyclic polyyne ring isomers⁹, like the bicyclic ring shown in Fig. 1. The drift-time distribution for LaC_{60}^+ has a narrow peak at $\sim 800 \mu\text{s}$ that we assign to the endohedral metallofullerene (see below) and a broad distribution of non-fullerene isomers extending out to drift times of $\sim 2,000 \mu\text{s}$. The distribution of non-fullerene isomers for LaC_{60}^+ is broader than observed for C_{60}^+ . The LaC_{60}^+ isomers with drift times of $\sim 1,300$ – $2,000 \mu\text{s}$ are probably due to La^+ attached to roughly planar polycyclic polyyne ring isomers similar to those observed for the pure carbon cluster. The LaC_{60}^+ isomers with drift times of ~ 800 – $1,300 \mu\text{s}$ are less compact than the metallofullerene, but more compact than the planar polycyclic rings. These are probably three-dimensional complexes of La^+ with polyyne rings, although the precise nature of the bonding is not known. The relative abundance of the non-fullerene LaC_{60}^+ isomers in Fig. 1 is $\sim 50\%$. The relative abundances of the different non-fullerene isomers were sensitive to the source conditions. The results shown in Fig. 1 are an average of three typical data sets.

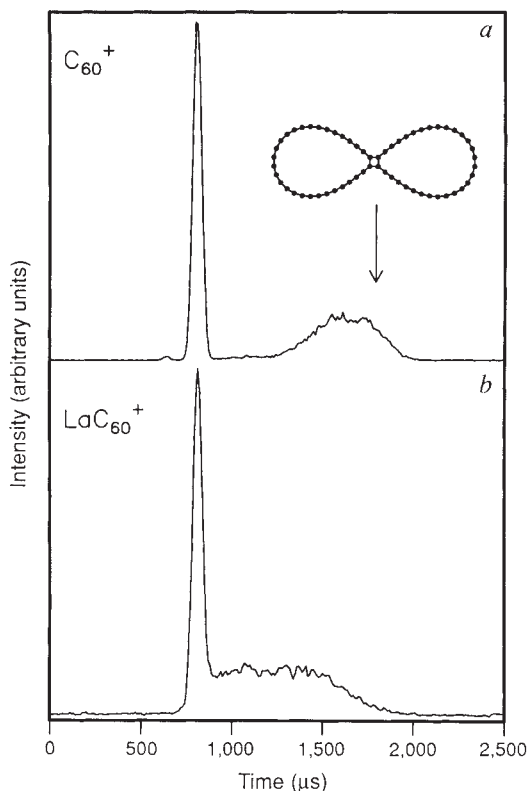


FIG. 1 Drift-time distributions recorded for a C_{60}^+ with an injection energy of 100 eV, and b, LaC_{60}^+ with an injection energy of 50 eV. The drift tube is 7.62 cm long and the drift field was 13.12 V cm^{-1} . The drift-time distributions have been scaled to a helium buffer gas pressure of 5.00 torr. With the injection energies employed here the isomer populations essentially reflect those generated by the source. The mass spectrum generated by laser vaporization of the La_2O_3 /graphite rod is dominated by peaks due to LaC_n^+ and C_n^+ . However, measurements of the isotope distributions show that the peaks were not entirely pure LaC_{60}^+ . Studies were performed as a function of isotopic composition and we are confident that the data presented here result from ions which are predominantly (>95%) LaC_{60}^+ .

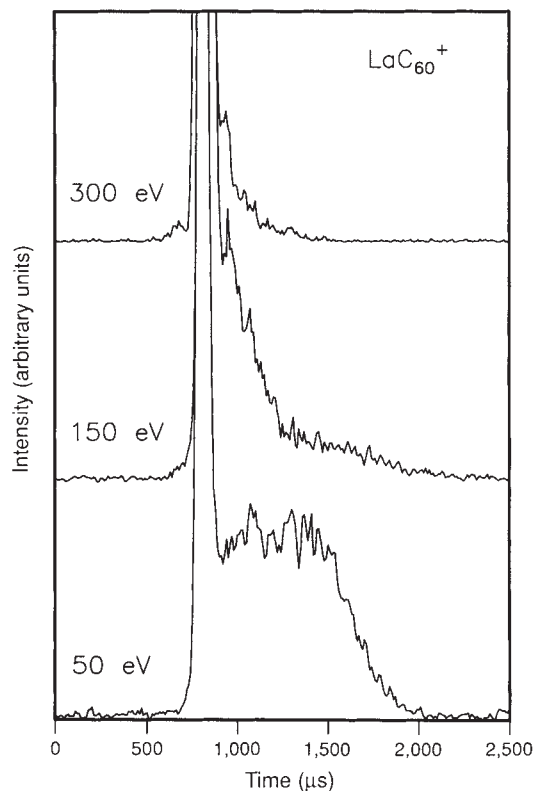


FIG. 2 Drift-time distributions recorded for LaC_{60}^+ as a function of injection energy (300, 150 and 50 eV). The experimental conditions were identical to those in Fig. 1.

product observed was La^+ with a relative abundance of <1%. Thus it appears that nearly all the non-fullerene LaC_{60}^+ isomers anneal into metallofullerenes.

Figure 4 shows the drift-time distribution measured for

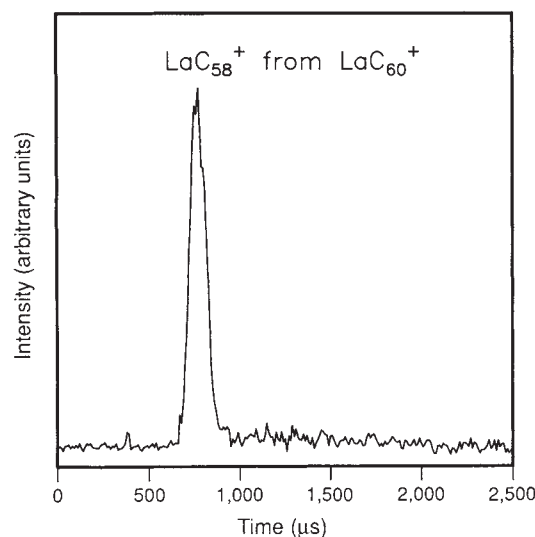


FIG. 3 Drift-time distribution recorded for LaC_{58}^+ resulting from LaC_{60}^+ injected into the drift tube at 200 eV. The experimental conditions were identical to those in Fig. 1. The only other product observed (except for the C_2 loss channels) was La^+ . La^+ is substantially lighter than LaC_{60}^+ , so there is a concern that the La^+ product could be discriminated against. Although there is no evidence for discrimination against the lighter products in previous studies⁸, experiments were performed as a function of buffer gas pressure to be sure that any La^+ formed before the entrance to the drift tube was not scattered away by collisions with the buffer gas. In all cases, the relative abundance of La^+ was <1%.

To anneal the clusters, the injection energy is increased so that the ions experience a rapid transient heating cycle as their kinetic energy is thermalized by collisions with the buffer gas. The ions are then rapidly cooled by further collisions with the buffer gas. Any fragmentation that occurs is monitored using a second quadrupole mass spectrometer after the drift tube. Figure 2 shows drift-time distributions recorded for LaC_{60}^+ as a function of injection energy. As the energy is increased to 150 eV the relative abundance of isomers with drift times >1,300 μs (the roughly planar polycyclic ring isomers) rapidly diminishes, and at the same time there is an increase in the relative abundance of non-fullerene isomers with shorter drift times. As the injection energy is raised further, the relative abundance of the non-fullerene isomers with shorter drift times decreases. The polycyclic polyene rings for LaC_{60}^+ anneal at lower injection energies than analogous C_{60}^+ isomers, indicating that the activation energy for annealing is lower for LaC_{60}^+ than for C_{60}^+ .

Not all the LaC_{60}^+ isomers anneal into an intact metallofullerene. At high injection energies (300–400 eV), up to ~10% of the non-fullerene isomers fragment to products which appear to result from sequential loss of C_2 units. Figure 3 shows the drift-time distribution recorded for LaC_{58}^+ resulting from injection of LaC_{60}^+ at 200 eV. These results demonstrate that the fragment ions are entirely metallofullerenes. A careful search was performed for other fragment ions. The only other dissociation

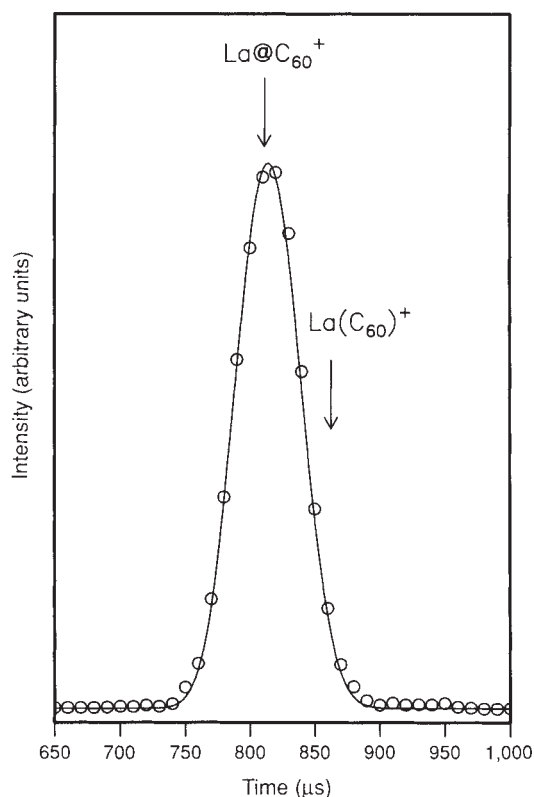


FIG. 4 Drift-time distribution (circles) recorded for LaC_{60}^+ with an injection energy of 400 eV. The solid line shows the drift-time distribution calculated from the transport equation for ions in the drift tube (see text). The arrows show the expected drift times for an endohedral La@C_{60}^+ and exohedral $\text{La}(\text{C}_{60})^+$ complex. The geometry of the exohedral $\text{La}(\text{C}_{60})^+$ was assumed to be a La atom sitting 2.4 Å from an unperturbed C_{60} . The mobility was calculated using a simple hard-sphere collision model¹⁰ (details are given elsewhere¹¹).

LaC_{60}^+ with an injection energy of 400 eV. At this high injection energy, all the non-fullerene isomers have annealed (or dissociated). The solid line in Fig. 4 shows the drift-time distribution calculated from the transport equation for ions in the drift tube¹⁰. The excellent agreement with the experimental results suggests that only a single LaC_{60}^+ isomer remains. Because the geometries of $\text{La}^+\text{@C}_{60}$ and $\text{La}(\text{C}_{60})^+$ are known, accurate drift times can be calculated from momentum transfer theory¹⁰. Arrows show the expected drift times¹¹ for an endohedral metallofullerene (which is assumed to be the same as for fullerene C_{60}^+) and an exohedral complex (where the lanthanum atom sits outside the fullerene cage). The measured drift time is in excellent agreement with that expected for the endohedral metallofullerene. The fragment ions (such as LaC_{58}^+) also appear to be endohedral metallofullerenes. A third isomer, where the lanthanum atom is incorporated into the fullerene cage, is also possible. Although the geometry of this isomer is not well established, we estimate that the drift time for this species falls roughly half way between the arrows shown in Fig. 4. Our data show no signs of this isomer.

The remarkably high efficiency with which the non-fullerene LaC_{60}^+ isomers anneal into endohedral metallofullerenes indicates that there must be a mechanism by which the lanthanum atom is trapped inside the annealing fullerene. The distribution of non-fullerene LaC_{60}^+ ions shifts to shorter times as the clusters are annealed, indicating that in the first steps of the annealing process the LaC_{60}^+ complexes become more compact. The opposite behaviour is observed in the annealing of pure C_{60}^+ , where the distribution of polyyne rings shifts to longer times as the polycyclic rings open to form bicyclic and monocyclic rings

before forming the fullerene⁶. Because the ionization energy of La (5.577 eV)¹² is substantially lower than that of fullerene C_{60} (7.62 eV)¹³, the charge is probably localized on the lanthanum. Therefore, a plausible explanation for the behaviour of the non-fullerene LaC_{60}^+ isomers is that the La^+ acts as a nucleation centre, and the carbon rings arrange themselves around the La^+ so that when they anneal into the fullerene cage the lanthanum atom is naturally encapsulated. Electrostatic interactions may play an important role in arranging the carbon rings in the early stages of the annealing process. This simple picture of the annealing mechanism accounts for both the shift to shorter times and the high efficiency of endohedral metallofullerene formation. □

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Quantitative self-assembly of a [2]catenane from two preformed molecular rings

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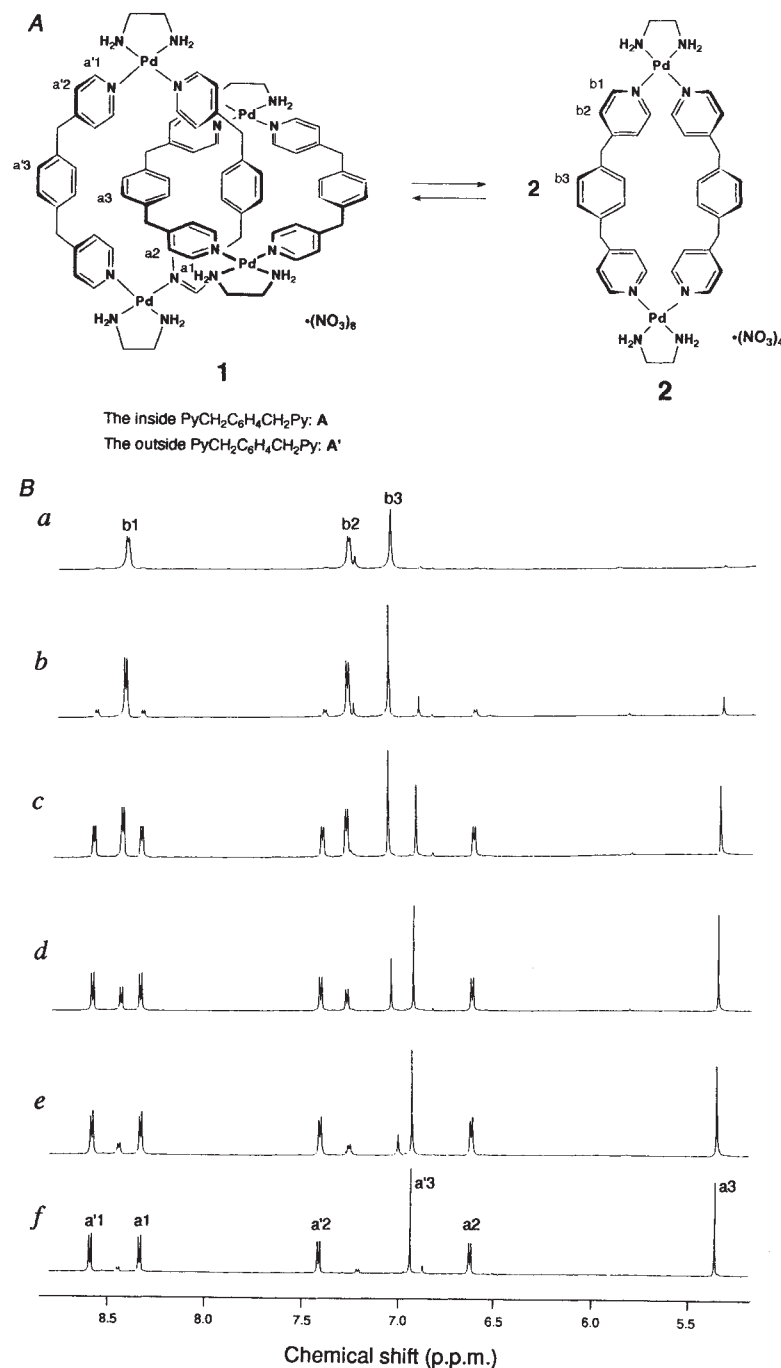
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MOLECULES composed of interlocking rings are termed catenanes. Since the first synthesis of a two-ring catenane (a [2]catenane) in 1960¹, these and related interlinked compounds have attracted considerable interest^{2–10}, in part from the perspective of forming molecular devices and nanoscale architectures^{11,12}. Here we report the formation of a [2]catenane from two complete rings, reminiscent of the trick of interlinking 'magic rings'. This supermolecule, 1 in Fig. 1, exists in rapid equilibrium with the monomeric rings 2, as revealed by ¹H NMR and electrospray mass spectrometry. At low concentration of molecule 2, the equilibrium lies towards the monomers, but at higher concentrations the catenane is the overwhelmingly dominant species in solution.

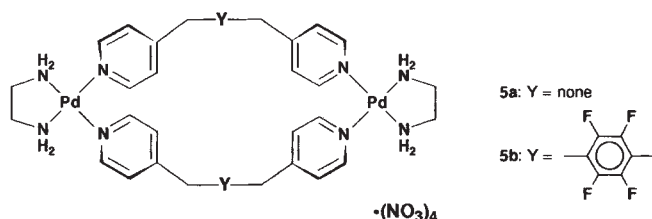
Previous syntheses of catenanes have all taken the approach of threading a ring on a thread and then linking the ends of the thread. In the syntheses of Stoddart and coworkers^{3–6}, for example, the ring and thread self-assemble so that the ends of the thread lie adjacent to one another and can readily be capped by a bridging unit. Our present approach exploits the relatively labile nature of metal–ligand bonds, which comprise a part of the rings, to interlock two preformed rings in solution.

When a D_2O solution of the Pd(II) complex $(\text{en})\text{Pd}(\text{NO}_3)_2$ (en , ethylenediamine) (3) (1 mM) was treated with the 1.0 molar equivalent 1,4-bis[(4-pyridyl)methyl]benzene (4), ¹H NMR

FIG. 1 A, Equilibrium between the catenane **1** and the monomeric ring **2**, shown (B) by ^1H NMR spectroscopy (aromatic region, 25°C , D_2O). Signals a1–a3 and a'1–a'3 refer to the inside (**A**) and the outside (**A'**) units of the catenane **1**, respectively. Signals b1–b3 refer to the ring **2**. Molecular modelling showed that the steric demand of the (en)Pd moiety inhibits an exchange between **A** and **A'** units, making them nonequivalent. Net Pd(II) concentrations (the concentrations of the components are given with those of net Pd(II) throughout this work): Ba, 1 mM; Bb, 2 mM; Bc, 5 mM; Bd, 10 mM; Be, 20 mM; Bf, 50 mM. These spectra were collected on separate solutions. A singlet signal at $\delta = 7.3$ in spectra Ba and Bb is due to the small quantity of CHCl_3 contained in the external TMS/CDCl_3 solution. Spectroscopic data of **1**: ^1H NMR (D_2O , TMS/ CDCl_3 as external standard; s, singlet; d, doublet; J, coupling constant) δ 2.83 (s, 16 H, $-\text{NCH}_2\text{CH}_2\text{N}-$ (**A**, **A'**)), 3.04 (s, 8 H, CH_2 (**A**)), 3.93 (s, 8 H, CH_2 (**A'**)), 5.34 (s, 8 H, C_6H_4 (**A**)), 6.59 (d-like, $J = 6.6$ Hz, 8 H, PyH_β (**A**)), 6.91 (s, 8 H, C_6H_4 (**A'**)), 7.38 (d-like, $J = 6.6$ Hz, 8 H, PyH_β (**A'**)), 8.30 (d-like, $J = 6.6$ Hz, 8 H, PyH_α (**A**)), 8.55 (d-like, $J = 6.6$ Hz, 8 H, PyH_α (**A'**)). ^{13}C NMR (D_2O , TMS/ CDCl_3 as an external standard) δ 39.57 (CH_2), 39.80 (CH_2), 46.65 (CH_2), 46.73 (CH_2), 126.56 (CH), 127.27 (CH), 128.45 (CH), 129.72 (CH), 135.42 (Cq), 136.22 (Cq), 150.67 (CH), 150.78 (CH), 154.98 (Cq), 156.24 (Cq). Spectroscopic data of **2**: ^1H NMR (D_2O , TMS/ CDCl_3 as an external standard) δ 2.84 (s, 8 H, $-\text{NCH}_2\text{CH}_2\text{N}-$), 4.03 (s, 8 H, ArCH_2), 7.07 (s, 8 H, C_6H_4), 7.29 (d-like, $J = 6.6$ Hz, 8 H, PyH_β), 8.42 (d-like, $J = 6.6$ Hz, 8 H, PyH_α). ^{13}C NMR (D_2O , TMS/ CDCl_3 as an external standard) δ 39.95 (CH_2), 46.96 (CH_2), 127.19 (Cq), 127.22 (CH), 130.08 (CH), 136.59 (Cq), 150.91 (CH), 156.67 (Cq). Addition of NaClO_4 to an aqueous solution of **1** precipitated pale yellow powder whose composition agreed with the ClO_4^- salt of **1** (48% yield): melting point $235\text{--}237^\circ\text{C}$ (decomposition). Results of analysis: Calculated for $\text{C}_{40}\text{H}_{48}\text{Cl}_4\text{N}_8\text{O}_{16}\text{Pd}_2$: C, 38.59; H, 4.09; N, 9.20; found: C, 38.39; H, 3.87; N, 8.95. Fast-atom-bombardment mass spectrometry (based on ^{106}Pd , glycerol): Mass-to-charge ratio m/z 1,149 (atomic mass units, a.m.u.) ($\text{M}-\text{ClO}_4$), 1,049 a.m.u. ($\text{M}-\text{ClO}_4-\text{HClO}_4$), 949 a.m.u. ($\text{M}-\text{ClO}_4-(\text{HClO}_4)_2$) (M denotes the parent molecule, in this case, **2**; see the result of run 13 of Table 1).

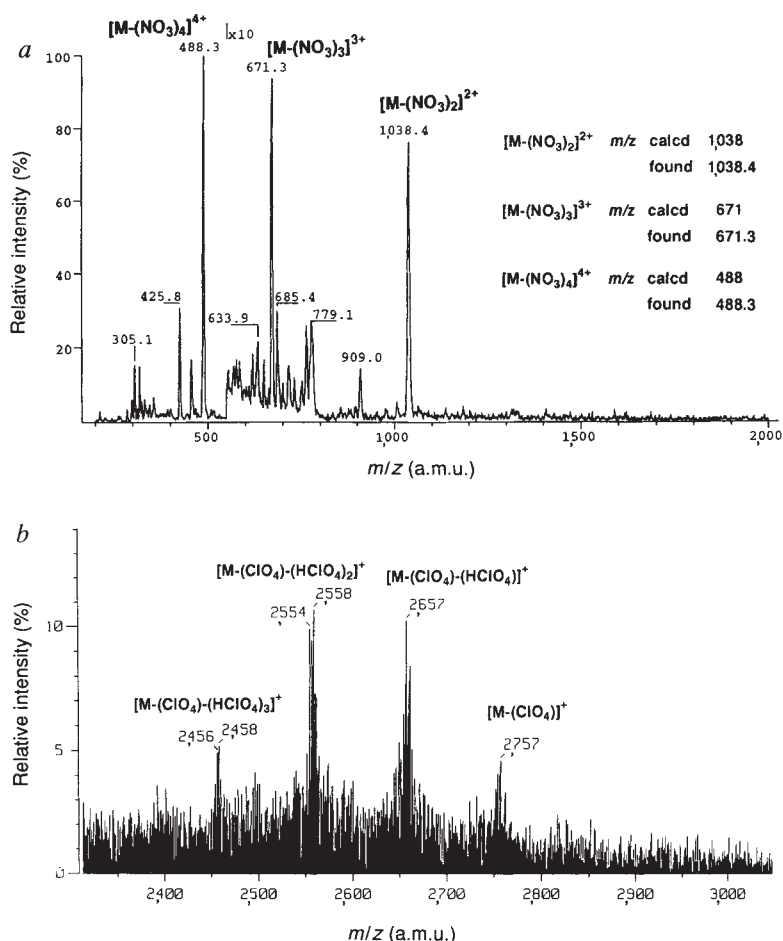


revealed the formation of a single component (Fig. 1Ba). We assign this component to the macrocyclic complex **2** by elemental analysis and fast-atom-bombardment mass spectrometry, as well as by similarities in NMR spectroscopy to the macrocyclic complexes, **5a,b** previously reported¹³. With increasing concentration, however, another component appeared (Fig. 1Bb–e); this component formed in high yields (>90%) at >50 mM (Fig. 1Bf).



That only catenane **1** fulfils the conditions for the structure of this component is concluded from the following careful consideration of spectroscopic observations: (1) Catenane **1** includes two nonequivalent $\text{PyCH}_2\text{C}_6\text{H}_4\text{CH}_2\text{Py}$ units referred to as **A** and **A'** (Fig. 1Bf); the assignments of which signals belong to **A** or **A'** were confirmed by NMR correlation spectroscopy through long-range coupling. (2) The very-high-field shift of $-\text{C}_6\text{H}_4-$ of **A** (a3) at $\delta = 5.3$ p.p.m. is characteristic^{6–8} of the inside unit that faces two aromatic rings of another ring (*H*, resonanced nuclei). (3) Strong nuclear Overhauser effects were observed between signals of $\text{C}_6\text{H}_4(\text{A})$ and $\text{C}_6\text{H}_4(\text{A}')$, $\text{C}_6\text{H}_4(\text{A})$ and $\text{PyCH}_2(\text{A}')$, $\text{PyCH}_2(\text{A})$ and $\text{C}_6\text{H}_4(\text{A}')$, and $\text{PyCH}_2(\text{A})$ and $\text{PyCH}_2(\text{A}')$. (4) Because catenane **1** has twice the molecular weight of **2**, the equilibrium ratio of **1** increases with increasing concentration. (5) More significantly, the electrospray mass spectrum of this component was consistent with the formula of **1** (Fig. 2a). A possibility of the formation of a cyclic tetramer with higher

FIG. 2 a, Electrospray mass spectrum of catenane **1**. Both calculated and observed mass-to-charge ratios (m/z) for prominent peaks derived from parent **1** are indicated. The spectrum was obtained on a TSQ700 spectrometer with electrospray ionization source (Finnigan MAT, San Jose, California) with an m/z range of 2,000. The electrospray interface was heated to 100 °C, the sampling cone voltage was 31.3 V and an aqueous solution of **1** (50 mM) was injected into the spectrometer. The flow rate was 2.00 $\mu\text{L min}^{-1}$. b, Fast-atom-bombardment mass spectrum of catenane **7**. The spectrum was obtained on a JEOL JMS-HX110A spectrometer with an m/z range of 3,000 using glycerol as matrix. Observed isotopic distribution of each fragment was in good agreement with theoretical one. (M denotes the catenane.)



restricted conformation is ruled out because conformational freeze should make the methylene protons (PyCH_2-) nonequivalent and should be released at elevated temperatures. However, the methylene protons are completely equivalent in high-resolution

NMR and the signals of A and A' did not coalesce even at 90 °C.

A control experiment with an analogous Pt complex, (en)Pt(NO₃)₂ (**6**), also confirmed the formation of catenane **1**. It is known that a pyridine nucleus coordinates to Pt(II) irreversibly at ambient temperature¹⁴. Thus, treatment of **6** (70 mM) with **4** (70 mM) afforded kinetically distributed products consisting of two main products assigned as catenane **7** and monomeric ring **8**, which are Pt analogues of **1** and **2**, respectively, in a 1:4 ratio (total ~60%) along with oligomeric components (~40%). Most significantly, dilution of the mixture did not affect the product ratio at all, in contrast to the behaviour of the Pd complexes. This fact indicates that the equilibrium observed for the Pd complexes is accomplished by breaking and reforming of the Pd-N(pyridine) bond.

Furthermore, catenane **7** was formed in a high yield by employing a more polar medium (5 M NaNO₃ aqueous solution), isolated as its ClO₄²⁻ salt, and characterized by NMR and fast-atom-bombardment mass spectrometry (Fig. 2b). Thus, the reaction of **6** (50 mM) and **4** (50 mM) in 5 M aqueous NaNO₃ afforded mainly **7** and **8** in a ~9:1 ratio (the medium effect will be discussed later). Subjection of the crude product to preparative thin layer chromatography (silica gel, 1 M aqueous NaNO₃-MeOH 1:3) followed by reprecipitation with aqueous NaClO₄ gave catenane **7** (ClO₄²⁻ salt) in 30% yield. Spectroscopic data (¹H and ¹³C NMR) of **7** were quite similar to those of **1**.

We suggest that the assembly of catenane **1** is promoted by 'double-differential complexation', in which two molecules of **2** bind each other in their cavities. The ability of **2** to bind with aromatic compounds was shown by ¹H NMR titration experiments¹⁵ at low concentrations: for example, the associa-

TABLE 1 Medium effect of the equilibrium between **1** and **2**

Run	Medium	Guest 9 *	Ratio† of 1 : 2
1	1.0 M NaNO ₃ /D ₂ O‡	—	>99:<1
2	0.2 M NaNO ₃ /D ₂ O	—	95:5
3	0.05 M NaNO ₃ /D ₂ O	—	86:14
4	0.01 M NaNO ₃ /D ₂ O	—	73:27
5	D ₂ O	—	59:41
6	D ₂ O	0.3	39:61
7	D ₂ O	0.5	27:73
8	D ₂ O	2.0	12:88
9	D ₂ O-CD ₃ OD (9:1)	—	44:56
10	D ₂ O-CD ₃ OD (7:3)	—	9:91
11	D ₂ O-CD ₃ OD (5:5)	—	<1:>99
12	D ₂ O-CD ₃ OD (5:5)§	—	<1:>99
13	D ₂ O-glycerol (5:5)	—	<1:>99

The structures of compounds **1** and **2** are shown in Fig. 1A.

*Molar equivalent to Pd(II).

† Measured at 10 mM unless otherwise noted.

‡ High yield formation of catenane **7** in 5 M NaNO₃ is also explained by this medium effect.

§ Measured 5 min after diluting the D₂O solution of **1** (50 mM) with an equal volume of CD₃OD.

|| This result was consistent with fast-atom-bombardment mass spectra (with glycerol matrix) which showed the fragments derived from only **2**.

tion constant $K_a = 201 \text{ l mol}^{-1}$, $\Delta G = 3.1 \text{ kcal mol}^{-1}$ for 1,4-dimethoxybenzene; $K_a = 200 \text{ l mol}^{-1}$, $\Delta G = 3.1 \text{ kcal mol}^{-1}$ for 1,4-dicyanobenzene in D_2O at 25°C . Double-differential complexation can produce approximately double the magnitude of ΔG , making the structure of **1** stable enough to assemble quantitatively at high concentration.

Such an interpretation is consistent with remarkable medium effect that enabled the equilibrium ratio **1**:**2** to be varied over the range of >99 : <1 to <1 : >99 (Table 1). Thus the use of a more polar median (D_2O solution of NaNO_3) increased the ratio of **1** up to $>99\%$ even at a low concentration, owing probably to enhanced hydrophobic interaction in the catenane formation (runs 1–4). In contrast, the ratio of **1** diminished with a less polar medium (CD_3OD – D_2O , runs 9–11). Selective stabilization of **2** by adding sodium (*p*-methoxyphenyl)acetate (**9**), a specific guest for **2**, also reduced the ratio **1**:**2** (runs 6–8). □

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An oscillation in the global climate system of period 65–70 years

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In addition to the well-known warming of $\sim 0.5^\circ\text{C}$ since the middle of the nineteenth century, global-mean surface temperature records^{1–4} display substantial variability on timescales of a century or less. Accurate prediction of future temperature change requires an understanding of the causes of this variability; possibilities include external factors, such as increasing greenhouse-gas concentrations^{5–7} and anthropogenic sulphate aerosols^{8–10}, and internal factors, both predictable (such as El Niño¹¹) and unpredictable (noise^{12,13}). Here we apply singular spectrum analysis^{14,20} to four global-mean temperature records^{1–4}, and identify a temperature oscillation with a period of 65–70 years. Singular spectrum analysis of the surface temperature records for 11 geographical regions shows that the 65–70-year oscillation is the statistical result of 50–88-year oscillations for the North Atlantic Ocean and its bounding Northern Hemisphere continents. These oscillations have obscured the greenhouse warming signal in the North Atlantic and North America. Comparison with previous observations and model simulations suggests that the oscillation arises from predictable internal variability of the ocean–atmosphere system.

For prescribed greenhouse-gas (GHG) and anthropogenic sulphate-aerosol (ASA) radiative forcing, the global-mean temperature changes simulated by our simple hemispherically resolved climate/ocean model^{9,10} for a range of climate sensitivities, $\Delta T_{2\times}$, are compared to the IPCC-observed global-mean surface temperature changes over 1858–1992 (ref. 1); the $\Delta T_{2\times}$ which minimizes the r.m.s. error is determined. The ASA radiative forcing, characterized by its value in 1978, $\Delta F_{\text{SO}_4}(1978)$, is chosen to minimize the r.m.s. error between the simulated and observed interhemispheric temperature differences. The simulated global-mean temperature changes are then subtracted from the observed global-mean temperature changes to reveal what is not caused by GHG and ASA radiative forcing. The resulting detrended temperature changes (Fig. 1d) indicate the presence of a non-random, oscillatory component.

We use singular spectrum analysis (SSA)^{14,20} to investigate this oscillatory component. (See Fig. 1 caption for an explanation of the quantities used in the following discussion.) Figure

1a shows the normalized eigenvalues λ_k , together with the 95% confidence interval, $\delta\lambda_k = 2\lambda_k(2/N)^{1/2}$ (refs 15,16), where N is the length of the observational record (data set) in years. Modes 1 and 2 form a pair which contribute 28.7% of the total variance. Vautard and Ghil¹⁶ and Vautard *et al.*¹⁷ have argued that such an eigenvalue pair is indicative of an oscillation. The distance of modes 1 and 2 above the ‘noise floor’ also indicates that these modes are deterministic rather than stochastic^{16,17}. Figure 1b presents eigenvectors ρ_1 and ρ_2 , each of which exhibits a quasi-periodic structure. Figure 1c shows the time series for modes 1 and 2, $\hat{X}_1(t_j)$ and $\hat{X}_2(t_j)$, $j=2, \dots, N$. Figure 1d presents $\hat{X}_1(t_j) + \hat{X}_2(t_j)$ in comparison with the detrended temperature anomaly. It is seen that $\hat{X}_1(t_j) + \hat{X}_2(t_j)$ has an oscillatory structure, with a timescale $\tau \approx 65$ yr.

The oscillation revealed in Fig. 1 has not been reported in the previous SSA studies^{18–20} of the global surface temperature anomaly data of Jones *et al.*². This is because those analyses were performed on the non-detrended data. To demonstrate this, we applied SSA to the non-detrended data of Jones *et al.*; we also analysed the non-detrended IPCC data and obtained very similar results. Reference 18 attributed modes 1 and 2 to the trend. Our singular spectrum analysis shows instead that mode 1 primarily represents the trend and secondarily the oscillation, and *vice versa* for mode 2. When SSA is applied to such a non-detrended time series, the first mode must explain the dominating variance contributed by the trend (62%), thereby masking within it the smaller variance contributed by the oscillation (8.4%). In detrending the temperature anomaly data, we removed the need for mode 1 to explain the variance of the trend, thereby leaving it free, together with mode 2, to explain the oscillation. We have confirmed these results by applying SSA to a time series composed of a linear trend and a sine wave. Consequently, we conclude that in using SSA, any deterministic trend should first be removed from the time series.

We have also applied SSA to the observed temperature anomaly data sets of Jones *et al.*², Hansen and Lebedeff³, and Vinnikov⁴, each revised and updated through 1992. The $\hat{X}_1(t_j) + \hat{X}_2(t_j)$ for each data set displays an oscillatory character, with good agreement between the values for the data of Hansen and Lebedeff, and Vinnikov, and separately between IPCC and Jones *et al.*. The differences between $\hat{X}_1(t_j) + \hat{X}_2(t_j)$ for the two pairs of data sets reflect the differences between the temperature observations themselves. The first pair of data sets (Hansen and Lebedeff, and Vinnikov) consist of land surface-air temperatures alone and begin in 1880 and 1881, whereas the second pair (IPCC and Jones *et al.*) also include sea surface temperatures (SST) and begin in 1858 and 1854. Estimation of τ from these four data sets over 1881–1992 yields 65, 66, 70 and 69 yr, respectively. Because the four time series are not longer than 2τ , however, these estimates must be considered as provisional and subject to revision when longer records become available.

FIG. 1 *a-d*, Singular spectrum analysis (SSA) results for the IPCC global-mean temperature anomaly data¹, detrended for the global-mean response simulated by our simple climate/ocean model for GHG and ASA ($\Delta F_{\text{SO}_4}(1978) = -0.35 \text{ W m}^{-2}$) forcing, with $\Delta T_{2\times} = 2.21^\circ\text{C}$. See text for discussion of these results. Symbols used here are defined below.

METHODS. The eigenvalues λ_k and eigenvectors ρ_k , $k=1, \dots, M$, of the scaled autocovariance matrix, C , a Toeplitz matrix with first row,

$$C_{1,j+1} = \frac{1}{M} \frac{1}{N-l} \sum_{i=1}^{N-l} X_j X_{j+i},$$

$l=0, \dots, M-1$, have been determined, where $X_j = X(t_j) - \bar{X}$; $t_j = t_0 + j$; $j=1, \dots, N$; $\bar{X}$ is the average of the detrended temperature anomaly, $X(t_j)$; and $M-1$ is the maximum number of lags l . The eigenvalues λ_k , divided by the variance of X_j , give the contribution of each eigenvector ρ_k to the variance. The eigenvectors $\rho_{k,i}$, $i=1, \dots, M$, or empirical orthogonal functions (EOFs)¹⁶⁻¹⁸, represent the temporal structure of each mode k . The time series for mode k is given by

$$\hat{X}_k(t_j) = \frac{1}{j-1} \sum_{i=1}^{j-1} A_{k,j-i} \rho_{k,i}$$

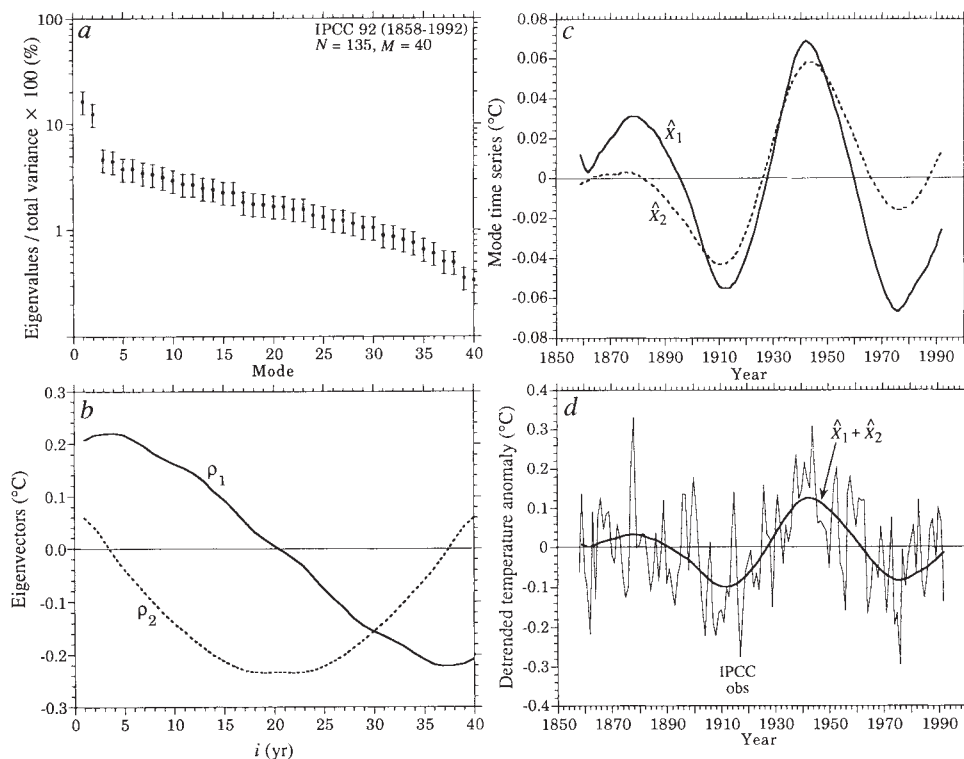
for $j=2, \dots, M$;

$$\frac{1}{M} \sum_{i=1}^M A_{k,j-i} \rho_{k,i}$$

for $j=M+1, \dots, N-M+1$, and

$$\frac{1}{M-j+1} \sum_{i=1}^M A_{k,j-i} \rho_{k,i}$$

for $j=N-M+2, \dots, N$, where



is the amplitude, or principal component (PC)¹⁶⁻¹⁸, of mode k at time t_j . For the IPCC data, $t_0 = 1857$, $N = 135$ and $\bar{X} = -4.67 \times 10^{-8}^\circ\text{C}$. Following refs 18-20, we choose $M = 40$; similar results are obtained for $30 \leq M \leq 50$.

To examine whether the oscillation exists globally or only in particular geographical regions, we subdivide the Earth's surface into 11 regions as shown in Fig. 2. For each region, a history of GHG + ASA-induced temperature change is obtained by area-weighting the surface-temperature changes simulated by our simple model for ocean and land in each hemisphere. This history is then subtracted from the Jones *et al.*² $5^\circ \times 5^\circ$ latitude-longitude gridded observed temperature anomaly data²¹, averaged for each region, and the resulting detrended record is analysed by SSA.

Figure 2 reveals an oscillation in the North Atlantic region with $\tau \approx 76$ yr, an explained variance for the detrended North Atlantic temperature record of 54.8%, and a correlation coefficient with mode 1 plus mode 2 of the detrended global-mean temperature record of 0.95. Long-timescale oscillations are also found for the Northern Hemisphere continental regions bounding the North Atlantic Ocean—North America, Eurasia and Africa—with $\tau \approx 88$, 84 and 50 yr, respectively. The regional eigenvalues, eigenvectors and principal components for modes higher than two (not presented) show that oscillations with timescales as long as these do not exist elsewhere. In the Southern Hemisphere regions there are shorter-timescale oscillations, with $9 \leq \tau \leq 20$ yr, the latter for the eastern equatorial Pacific and South Pacific regions. The regional eigenvalues, eigenvectors and principal components for modes higher than two show that such short-timescale oscillations also exist in Eurasia and Africa. Thus the 20-yr oscillation in global-mean temperature, whose existence has been argued in refs 18-20, does not exist everywhere.

Figure 2 suggests some residual trend for the South Atlantic region. This is a consequence of using the hemispheric-mean temperature changes simulated by our simple model, separately for land and ocean, to detrend the observed regional temperature anomalies. Eventually, improvements in detrending might be

achieved by using the geographical distribution of temperature change simulated by a calibrated atmosphere-ocean general circulation model for the time-dependent GHG and ASA forcing since 1850. It is preferable at present to apply SSA to an imperfectly detrended time series than to the non-detrended time series. The details of the regional oscillations shown in Fig. 2 should, however, be treated with caution.

The geographical structure of the oscillation is revealed in Fig. 3 by the product of the differences of 20-yr-averaged temperature anomalies, centred at the extrema of global-mean oscillation. The oscillation is seen to exist over the North Atlantic Ocean, North America, western Eurasia and northern Africa, and not to exist over most of the Southern Hemisphere where data exist back to 1900.

Figures 2 and 3 show that the 65-70-yr oscillation in global-mean temperature is the statistical result of the regional long-timescale oscillations which occur exclusively in the Northern Hemisphere. The 65-70-yr timescale of the irregular 'global' oscillation lies within the timescale band of these regional oscillations, 50-88-yr; the peak-to-peak range of the 'global' oscillation, 0.19°C , is reduced by 75% compared to the maximum regional peak-to-peak range, 0.75°C , for the North Atlantic region.

There are three possible sources for the 65-70-yr 'global' oscillation: (1) random forcing, such as by white noise; (2) external oscillatory forcing, such as by a variation in the solar constant; and (3) an internal oscillation of the atmosphere-ocean system. Atmospheric white-noise forcing of the ocean, evoking a red-noise response, was proposed by Hasselmann²² and has been invoked to explain the non-GHG + ASA component of the observed temperature record¹². Putative variations in the solar constant have also been proposed to explain this^{23,24,10}. But it is unlikely that either of these forcings is the source of the 65-70-

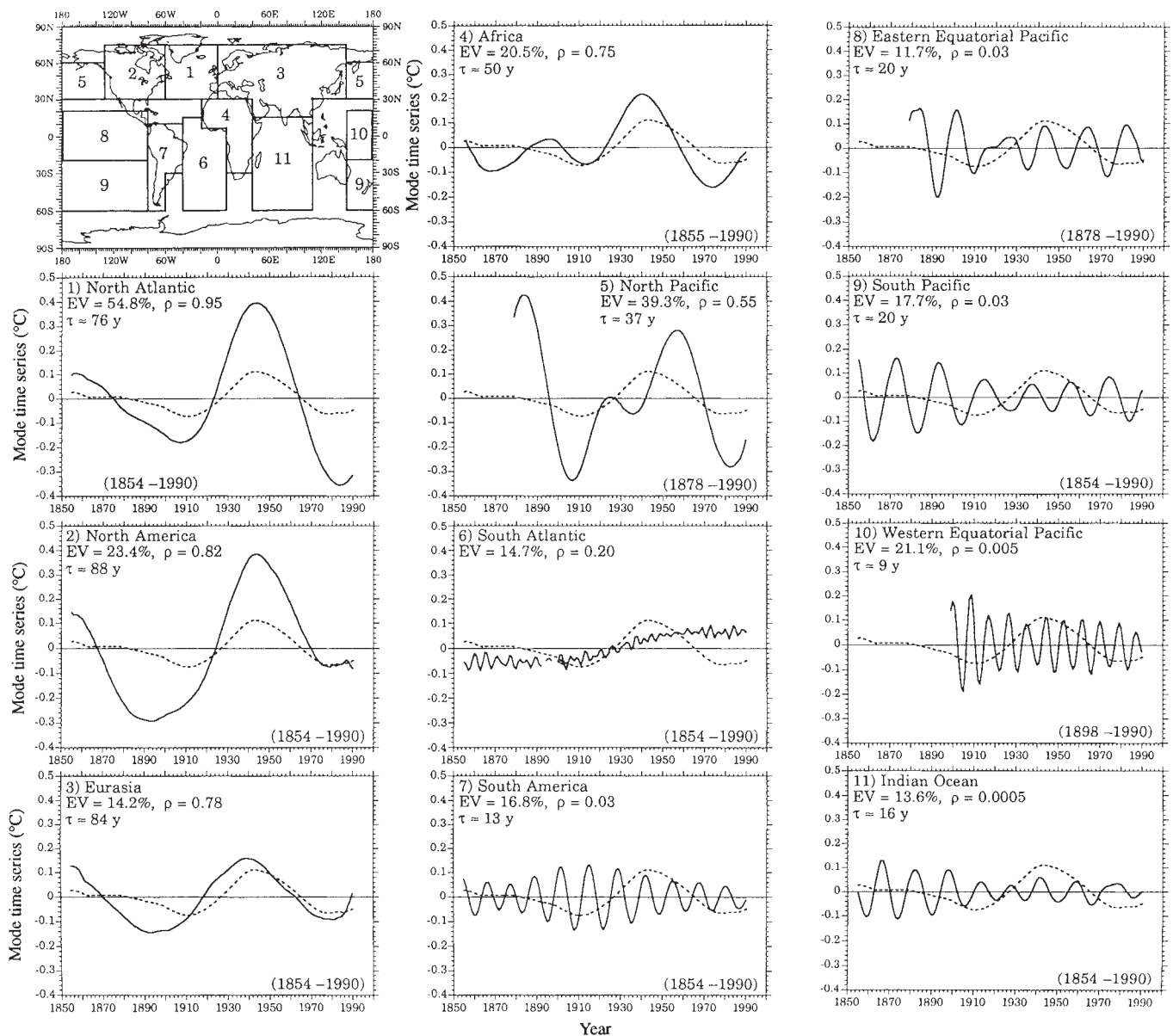


FIG. 2 SSA results for the Jones *et al.*² gridded temperature anomaly data²⁴ ($M=40$) for the 11 regions shown (top left), each detrended for its area-weighted land and ocean hemispheric response simulated by our simple climate/ocean model for GHG and ASA ($\Delta F_{\text{SO}_4(1978)} = -0.37 \text{ W m}^{-2}$) forcing, with $\Delta T_{2\times} = 2.58^\circ\text{C}$. (Each panel (1–11) corresponds to one of the 11 regions). The contribution to the detrended temperature record by the two SSA modes which explain the largest

amount of its variance, $\hat{X}_1 + \hat{X}_2$, is shown for each region (solid line) and for the Jones *et al.*² global-mean temperature anomaly (dashed line). Symbols used are as follows: the regional variance explained by the regional $\hat{X}_1 + \hat{X}_2$ is EV. The correlation coefficient between the regional and global-mean $\hat{X}_1 + \hat{X}_2$ is ρ . The timescale for the regional $\hat{X}_1 + \hat{X}_2$, τ , was estimated by the average time between consecutive maxima and between consecutive minima.

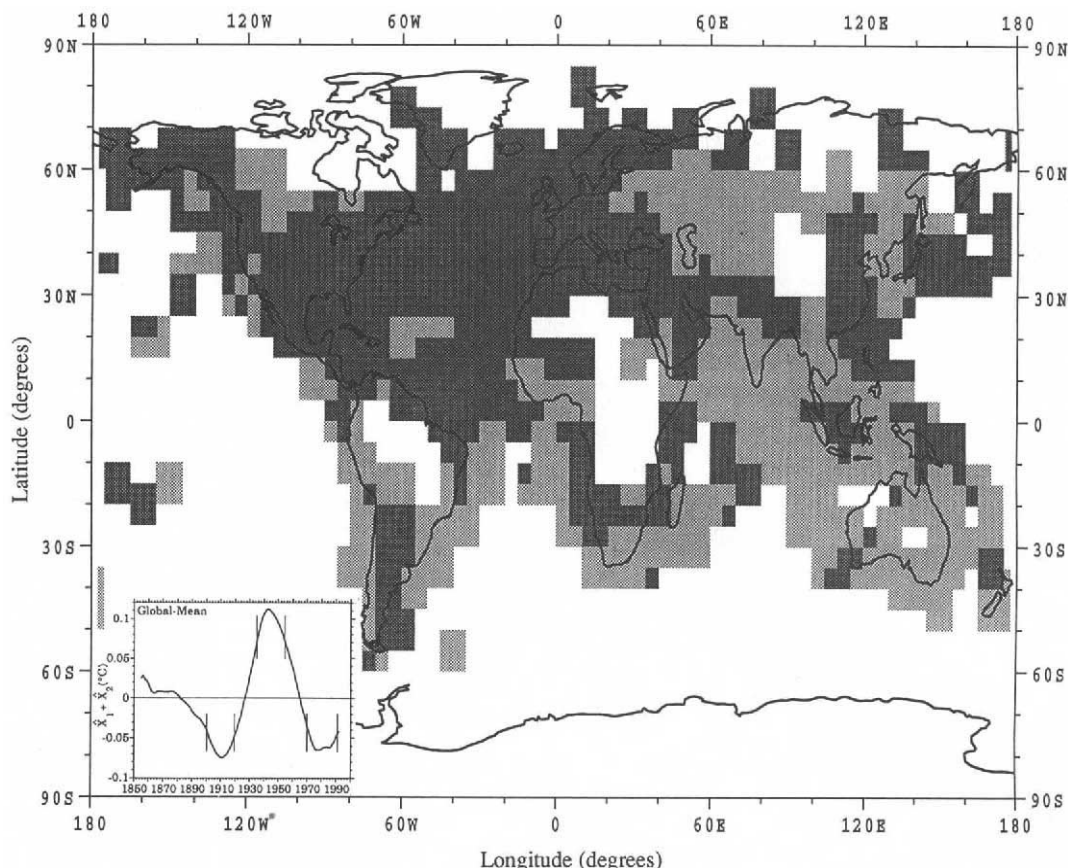
yr oscillation—solar forcing should generate a global response^{25,26} and white-noise forcing an ocean-wide response, but the 65–70-yr oscillation is neither global nor pan-oceanic. The most probable cause of this oscillation is therefore an internal oscillation of the atmospheric–ocean system.

This conclusion is supported by a growing body of observational evidence and model-simulation results. Bjerknes (1964)²⁷ analysed North Atlantic SST data spanning 1894–1934 and found basin-wide interdecadal differences. Levitus (1989)²⁸ analysed North Atlantic hydrographic temperature and salinity data spanning 1955–74 and found coherent interdecadal differences from the sea surface down to at least 2,000 m depth. Kushnir (1994)²⁹ analysed a century of SST data from the Comprehensive Ocean–Atmosphere Data Set for the North Atlantic and found a long-term fluctuation, with negative anomalies before 1920,

positive anomalies between 1930 and 1960, and cold anomalies in the 1970s and 80s. Delworth *et al.* (1994)³⁰ performed a 600-yr-long simulation with a global atmosphere–ocean general circulation model and obtained an irregular oscillation of SST and the thermohaline circulation in the North Atlantic having a time-scale of ~40–60 yr.

Comparison of the regional and global-mean temperature changes induced by GHG + ASA forcing with those caused by the oscillation shows the rapid rise in global-mean temperature between about 1908 and 1946, and the subsequent reversal of this warming until about 1965, were the result of the oscillation in regions 1–4 of Fig. 2. In the North Atlantic and North American regions the oscillation has so far dominated the GHG + ASA-induced warming, thereby obscuring the latter and confounding its detection³¹. It is likely that this North Atlantic

FIG. 3 Geographical distribution of the product of the differences of 20-yr-averaged temperature anomalies, centred at the extrema of global-mean oscillation (inset): $[(1900-20) - (1935-55)] \times [(1935-55) - (1970-90)]$. The negative (positive) product, shown by dark (light) shading, is where the temperature anomaly changed (did not change) from negative during 1900-20 to positive during 1935-55 and back to negative during 1970-90. In non-shaded regions there is no product because data are absent during at least one of the 20-yr averaging periods.



oscillation has also influenced past climates as revealed by the recent Greenland ice-core analyses³²⁻³⁴. It is to be hoped that the future course of this oscillation can be predicted by atmosphere-ocean general circulation models. □

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Relationship of baleen whales established by cytochrome *b* gene sequence comparison

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A RECENT revision¹ of whale phylogeny suggested that the sperm whale was more closely related to rorquals than to other toothed whales. This made the suborder Odontoceti (toothed whales) paraphyletic, and implied that the latest common ancestor of rorquals and sperm whales may have lived only 10-13 million years ago. This is at variance with palaeontological evidence for the greater antiquity for both mysticetes (baleen whales) and sperm whales, so the Mysticeti, as well as the Odontoceti, must also be paraphyletic if the dates implied in ref. 1 were correct. Here we present a more comprehensive phylogenetic analysis that demonstrates the monophyly of mysticetes and identifies no particular affinity between the sperm whales and rorquals.

The Odontoceti and the Mysticeti are generally considered to

constitute two well defined suborders of the order Cetacea. A separate position of the sperm whales within the Odontoceti has, however, been documented at both the DNA² and chromosome^{3,4} levels. The results are consistent with both morphological^{5,6} and palaeontological⁷ findings. Analyses of two sets of molecular data, myoglobin^{8,9} and partial mitochondrial (mtDNA) 16S ribosomal DNA (rDNA) sequences¹⁰, join the sperm whale and orqual to the exclusion of the family Delphinidae (dolphins). However, the myoglobin findings are not consistent with haemoglobin studies of the same taxa^{8,9}, and the myoglobin and 16S rDNA results have been considered anomalous when viewed in conjunction with the comprehensive body of evidence provided by other sets of data¹⁰.

Myoglobin data and partial sequence data of mitochondrial 12S and 16S rDNA were recently used for proposing a revised cetacean phylogeny¹. That study reported affinities between the sperm whales (family Physeteridae) and a particular mysticete family, the Balaenopteridae, orquals. On the basis of these findings, it was suggested that the evolutionary separation between the sperm whales and the orquals might have occurred as late as 10–13 Myr ago. The authors also presented “a phylogenetic hypothesis for all major groups of cetaceans (except river dolphins)”. That claim is questionable considering the fact that three mysticete families, Balaenidae, Neobalaenidae and Eschrichtiidae, were not represented by 12S and 16S data in the proposed revision¹ of cetacean phylogeny. The palaeontological record of the Physeteridae is ≥ 20 Myr¹¹. This figure is thus much higher than that given in ref. 1 for the separation of the Physeteridae and Balaenopteridae (see also ref. 12). The value of 10–13 Myr is also much lower than that of the primary evolutionary divergence of the mysticetes, namely the separation between the lineages of the Balaenidae (right whales) and the remaining families, Neobalaenidae, Eschrichtiidae and Balaenopteridae, which took place ≥ 17 Myr ago^{11,13,14}. If the claim is correct that the Physeteridae and the Balaenopteridae separated 10–13 Myr ago¹, then this separation must have occurred later than the separation between the lineages leading to the Balaenidae and Balaenopteridae, thereby rendering the Mysticeti paraphyletic. The palaeontological record of the Mysticeti was not

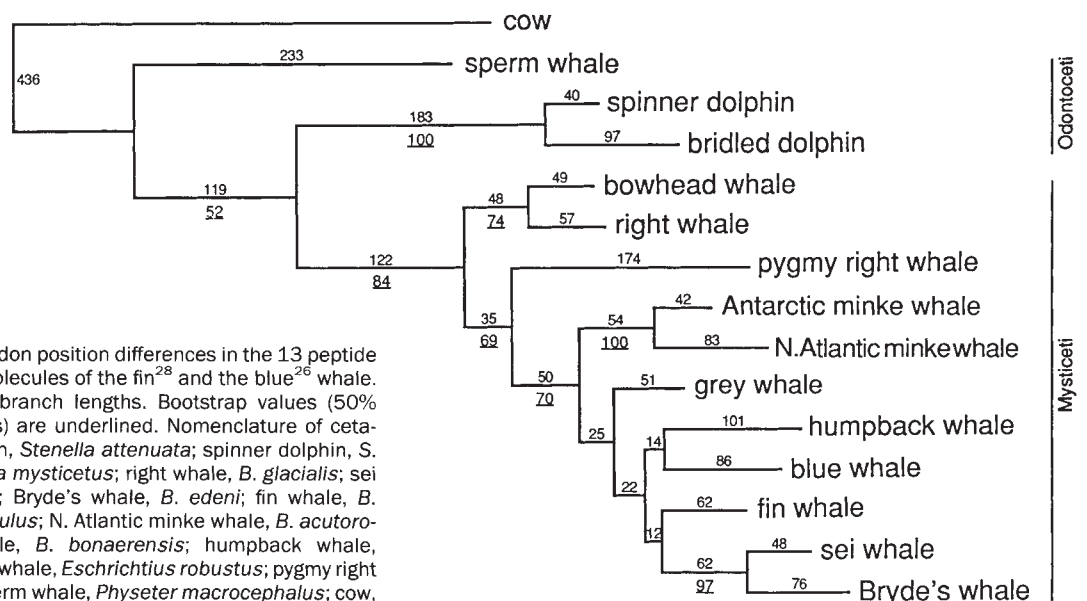
considered in depth in ref. 1 and the crucial implication of mysticete paraphyly was therefore not recognized.

Here we use the sequence of the complete cytochrome *b* gene of mtDNA to address evolutionary relationships within the suborder Mysticeti. We also examine the affinities between the sperm whale and the orquals, which formed the basis for the proposed revision of whale phylogeny¹.

Analysis (PAUP¹⁵) of 11 mysticete sequences, two dolphin sequences¹⁶, and that of the sperm whale (using cow sequence¹⁷ as outgroup) identified the Mysticeti as monophyletic. The comparison yielded two equally parsimonious trees that differed only with respect to the position of the grey whale. In the tree presented in Fig. 1, the grey whale is positioned between the minke whales and the remaining orquals; in the other arrangement (not shown) the grey whale was placed between the blue/humpback whales and the fin whale. The relationships between the grey, humpback, blue and fin whales were not resolved in the bootstrap analysis.

Within the Mysticeti the two Balaenidae species (bowhead, right whale) are separated from the remaining nine species. The latter clade includes the pygmy right, grey, humpback, blue, fin, sei and Bryde's whales, plus the two minke whale types. In this clade, the pygmy right whale is separated from the remaining eight sequences, that is, all orquals and the grey whale. Usually the pygmy right whale is included in the Balaenidae, but its placement in a separate family, Neobalaenidae, has been proposed on the basis of anatomical^{11,18,20} and molecular^{14,21–23} studies. The position of the grey whale among the orquals is notable. The palaeontological record of the Eschrichtiidae is very short, only ~100,000 years, and its evolutionary origin has not been conclusively settled¹³. Our findings and other molecular data (taxonomic distribution of the light mysticete satellite²¹, and sequence analyses of the common cetacean DNA satellite¹⁴, the heavy mysticete satellite²², and the stable portion of the mtDNA control region²³) all suggest a close relationship between the grey whale and the orquals. The findings exemplify, in a striking fashion, the rapidity at which morphological adaptation may occur when a new ecological niche is being occupied. The establishment of the evolutionary position of the grey whale

FIG. 1 Phylogenetic tree (heuristic search, maximum parsimony) showing the relationships between the nucleotide sequences of the cytochrome *b* gene (1,140 bp) of 11 mysticetes, 2 dolphins, and the sperm whale, using the cow as outgroup. Differences in codon positions 1, 2 and 3 were given the weight 4, 17 and 1, respectively. The allocated weight is based on all codon position differences in the 13 peptide coding genes of the mtDNA molecules of the fin²⁸ and the blue²⁶ whale. Values not underlined show branch lengths. Bootstrap values (50% majority rule, 1,000 replicates) are underlined. Nomenclature of cetacean species²⁹: bridled dolphin, *Stenella attenuata*; spinner dolphin, *S. longirostris*; bowhead, *Balaena mysticetus*; right whale, *B. glacialis*; sei whale, *Balaenoptera borealis*; Bryde's whale, *B. edeni*; fin whale, *B. physalus*; blue whale, *B. musculus*; N. Atlantic minke whale, *B. acutorostrata*; Antarctic minke whale, *B. bonaerensis*; humpback whale, *Megaptera novaeangliae*; grey whale, *Eschrichtius robustus*; pygmy right whale, *Caperea marginata*; sperm whale, *Physeter macrocephalus*; cow, *Bos taurus*. The sequences of the cow¹⁷, dolphins¹⁶, fin whale²⁸ and blue whale²⁶ are published. Other sequences were amplified by polymerase chain reaction (PCR) using a primer set based on the mtDNA sequence of the fin whale. The amplified products were cloned in M13mp18/19, except that of the Antarctic minke whale, which was sequenced directly. The sequences have been deposited at EMBL with accession numbers X75581–X75589, and X75753. The sequences of



cloned PCR products constitute the consensus of three clones. The comparison yielded two equally parsimonious trees which differed only with respect to the position of the grey whale. In the other tree (not shown), the grey whale was positioned between the fin and the blue/humpback whales.

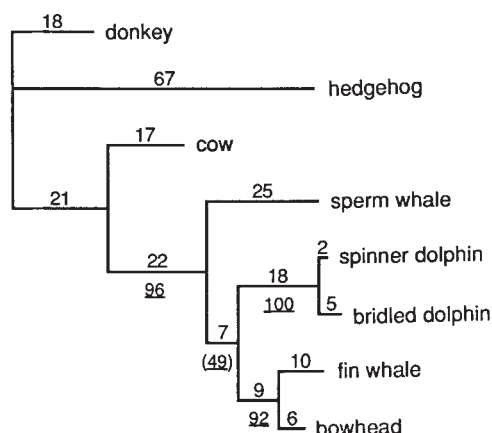


FIG. 2 Phylogenetic tree of a PROTPARS comparison between the inferred peptide sequences of cytochrome *b* of spinner dolphin, bridled dolphin, bowhead, fin whale, sperm whale and cow using hedgehog, *Erinaceus europaeus*, and donkey, *Equus asinus*, as outgroup. Branch lengths (not underlined values) and bootstrap values (underlined, 50% majority rule, 1,000 replicates) are included. When the cow was used as outgroup instead of the hedgehog and the donkey, the bootstrap value for the sperm whale versus the other cetacean species was raised marginally, from 49 to 53.

is of considerable interest for defining primitive and derived anatomical characters within the suborder Mysticeti.

The sei and Bryde's whale constitute the most closely related pair of mysticete species and the difference between the two species is actually considerably less than that between the fin and blue whales, two species that produce female hybrids which are not completely sterile^{24, 26}.

As shown in Fig. 1, the two minke whale types have a distant position among the rorquals. The difference between the two types is similar to, or even greater than, that between acknowledged mysticete species. The position of, and the difference between, the two types conforms with recent sequence comparisons of the control region of mysticete mtDNA and are consistent with the molecular view that the two types should have full species status, *Balaenoptera acutorostrata* (N. Atlantic minke whale) and *B. bonaerensis* (Antarctic minke whale)²³.

The difference between the two right whale species, the bowhead and the right whale, is less than that between some species of genus *Balaenoptera*. The results indicate that there is not a generic distinction between the two species and that they should be included in the same genus, *Balaena*.

As shown in Fig. 1, there are no particular affinities between the rorqual and sperm whale sequences and the sperm whale is more distant to the mysticetes than are the two dolphins. Thus the DNA analyses do not support a close relationship between the sperm whale and the rorquals. This particular relationship was addressed in a PROTPARS²⁷ comparison (PAUP) comprising sperm whale, two dolphins, fin whale, bowhead, and cow (Fig. 2). The hedgehog and donkey constituted outgroup species as compared with the sloth and the ass used in ref. 1. The bowhead was included in the analysis because both palaeontological and molecular data suggest that the rorquals and the right

whales separated much earlier than the suggested¹ 10–13 Myr separating the sperm whale and the rorquals, which implies Mysticeti paraphyly, as stated earlier. The dolphins and the baleen whales were joined in 49% of the bootstrap analysis. The corresponding figure for the dolphins and the sperm whale was 27%, and that for the sperm whale and the baleen whales, 22%. The bootstrap analysis yielded no support to the reported affinities between the sperm whale and the rorquals (bootstrap value 1%).

The mysticete relationships reported here are consistent with molecular results of two unrelated DNA satellites²¹, neither of which occur in sperm whale DNA. The data also agree with sequence analyses of the common cetacean satellite¹⁴, the heavy mysticete satellite²², and the mysticete mtDNA control region²³. The latter study also included the corresponding region of the sperm and pygmy sperm (*Kogia breviceps*) whales. Although the mysticete sequences are similar, pronounced differences occur between the mysticetes and the sperm whales.

The proposal for close affinities between the sperm whales and the rorquals and the recently suggested revision of cetacean phylogeny¹ is inconsistent with our results. It is also inconsistent with anatomical and palaeontological findings, cytogenetic studies and previous molecular analyses, all of which are compatible with a long separate evolution of the sperm whales. We therefore seriously challenge the proposed revision.

The DNA study in ref. 1 was based on partial sequence data of the two mitochondrial ribosomal genes (12S and 16S). In the case of the 12S gene the sequence was ~385 base pairs (bp). The full length of the cetacean 12S rDNA sequence is ~960 bp, and comparison between a complete 12S rDNA sequence of a dolphin, the sperm whale, and the fin whale does not support the close sperm whale/rorqual affinities reported in ref. 1 (E. Douzery, personal communication). □

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Dynamic neuromodulation of synaptic strength intrinsic to a central pattern generator circuit

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MOTOR circuits are often thought to be physically separate from their neuromodulatory systems^{1,2}. We report here a counter example, where neurons within a circuit appear to modulate synaptic properties of that same circuit during its normal operation. The dorsal swim interneurons (DSIs) are members of the central pattern generator circuit for escape swimming in the mollusc *Tritonia diomedea*³. However, DSI stimulation also rapidly enhances the synaptic potentials evoked by another neuron in the same circuit onto its follower cells. This modulatory action appears to be mediated by serotonin (5-hydroxytryptamine); the DSIs are serotonin-immunoreactive⁴, and bath-application of serotonin mimics and occludes the effect of DSIs. These results indicate that during the escape swim, circuit connection strengths are dynamically controlled by the activity of neurons within the circuit itself. This 'intrinsic neuromodulation' may be important for the animal's initial decision to swim, the generation of the swim motor programme itself, and certain types of learning.

The DSI neurons (DSI-A, B and C) are essential elements of the central pattern generator (CPG) circuit for escape swimming in *Tritonia* (Fig. 1a). Through their synaptic connections with the ventral swim interneurons (VSI) and C2 cerebral neurons,

they participate in the generation of the swim motor programme⁵ (Fig. 1b); brief hyperpolarization of several DSIs can reset the rhythm³, whereas depolarization of individual DSIs can prolong the motor pattern⁵. In previous considerations of this network, no other role was suggested for the DSIs and no heterosynaptic changes of synaptic efficacy were known to occur during the behavioural response. We show here that the DSI cells have an additional powerful neuromodulatory role, increasing the gain of specific network synapses, during the normal operation of the circuit⁶.

Stimulating individual DSIs at rates similar to those observed during a swim episode (Fig. 1b) enhanced the strength of synapses made by the CPG interneuron C2 onto its postsynaptic followers in the swim motor network, both inside and outside the CPG (Fig. 2A, B). Within the CPG circuit, C2 evokes a dual-component synaptic potential in DSI-B (Fig. 2A, a), consisting of an initial excitatory component followed by a prolonged inhibitory potential⁷. When the C2-DSI connection was examined either during or immediately after stimulation of any one of the other DSI neurons, the size of the inhibitory synaptic potential was significantly enhanced (Fig. 2A, a). DSI stimulation increased the amplitude of the inhibitory component an average of $71 \pm 16\%$ (Fig. 2A, b). The excitatory component was also enhanced in some, but not all, cases.

DSI stimulation also increased the synaptic strength of other C2 synapses onto neurons within the same motor circuit, but outside the CPG. DSI stimulation increased the amplitude of the excitatory postsynaptic potential (e.p.s.p.) evoked by C2 in the dorsal flexion neuron (DFN) by an average of $198 \pm 21\%$ (Fig. 2B).

The modulation of C2 synaptic strength was quite dynamic, arising within seconds of the onset of DSI stimulation and decaying within a few seconds of its cessation (Fig. 2C, D). In three animals for which identical stimulus protocols were used, modulation of the C2-DFN e.p.s.p. decayed to a half-maximum value 5.7 s after the end of the DSI spike train (5 Hz for 10 s).

Previous work reported that the DSI neurons are immuno-

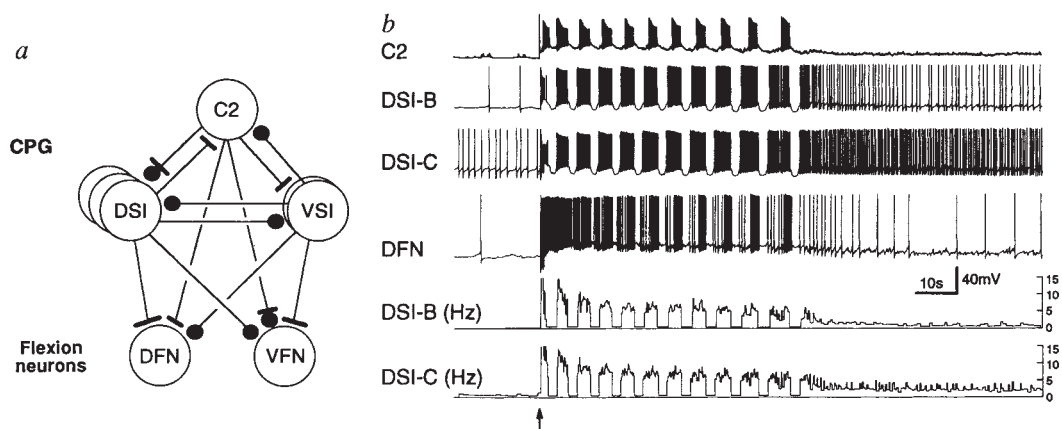


FIG. 1 The organization and neural firing pattern of the *Tritonia* swim motor network. a, The central pattern generator (CPG) circuit⁷, consisting of the dorsal swim interneurons (DSI), ventral swim interneurons (VSI), and cerebral neuron 2 (C2), generates the swim behaviour^{3,5,7,9,24}. The CPG interneurons synapse on two classes of efferent neurons: the dorsal flexion neurons (DFN) and the ventral flexion neurons (VFN) which drive the muscle movements²⁵⁻²⁷. There are two types of DFN (DFN-A and DFN-B); only DFN-A will be discussed here. The monosynaptic connections in the CPG between the three DSIs (DSI-A,B,C), the two VSIs, and the single C2 are shown for one side of the brain⁷. Excitatory connections are denoted by bars, inhibitory by circles, and multicomponent synapses by combinations of the two. C2 makes a biphasic excitatory/inhibitory connection onto DSI-B and -C, but is purely inhibitory onto DSI-A (our unpublished data, and ref. 7). b, The swim motor pro-

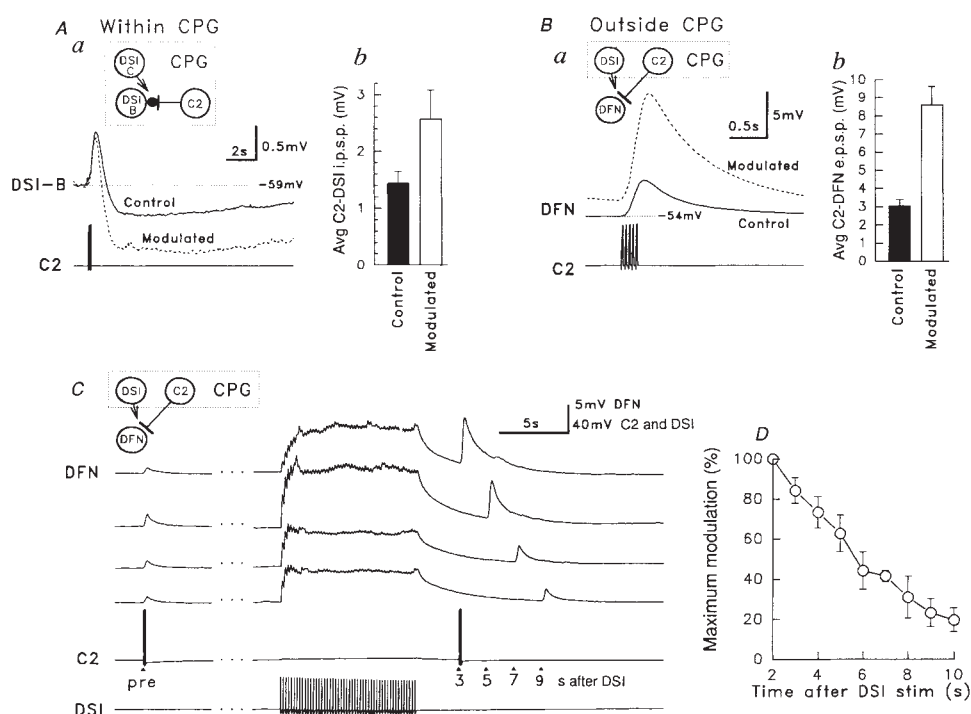
gramme was elicited in an isolated brain preparation by brief stimulation of pedal nerve 3 (10 Hz, 1 s at arrow). Simultaneous intracellular recordings are shown from all of the neurons described in this study (C2, two DSIs and a DFN) using glass microelectrodes (10–20 MΩ) filled with 4 M potassium acetate. All neurons were identified on the basis of soma location and coloration, synaptic interactions, and activity pattern during the swim motor programme^{3,25}. Instantaneous spike frequency profiles are shown in the bottom two traces for the DSI neurons. Note that the spontaneous spike activity of both DSIs following the swim was greater than that before the swim. Normal saline composition (in mM): 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 10 HEPES (pH 7.6), 11 D-glucose. Animals were collected from four different locations in the coastal waters off California, Washington and Vancouver Island.

reactive for serotonin (5-HT)⁴. We have replicated this finding (Fig. 3a, b) and have obtained further evidence suggesting that DSI may release 5-HT to produce its modulatory effects. Like DSI stimulation, bath-application of 5-HT (100 μ M) increased the amplitude of the synaptic potentials evoked by C2 (Fig. 3C–E). This 5-HT-evoked enhancement of C2 synapses was not additive with the effect of DSI stimulation, and in fact completely occluded the modulatory action of DSI in each of the three experiments in which it was tested (Fig. 3F). These data, together with other data on the effects of 5-HT antagonists and precursors⁸, are consistent with the hypothesis that DSI produces heterosynaptic facilitation of C2 synapses by releasing 5-HT.

The function of this intrinsic neuromodulation is not yet known, but there are many intriguing possibilities. For example, it may be involved in the animal's decision to swim; previous work has established that, at rest, these neurons are components of a reflex withdrawal circuit which rapidly reconfigures into a CPG circuit in response to an appropriately strong stimulus^{9–11}. As this CPG is a network oscillator dependent upon synaptic properties to produce its rhythmic output^{5,9}, the rapid modulation of C2 synapses within the CPG may be an important component of the reconfiguration mechanism. Modulation of the C2 connections outside the CPG may also be important in the overall reconfiguration mechanism, enhancing the effectiveness of the CPG at driving the efferent neurons. In addition, the rapid onset and decay of the modulation indicates that it may rise and fall cycle by cycle during the swim itself, suggesting a possible role in the actual mechanics of rhythm generation. Finally, the modulation may have a role in non-associative learning; following a swim episode, the spontaneous firing frequency of all DSIs remains elevated for several minutes (Fig. 1b). As a single DSI firing at frequencies as low as 2 Hz is able to enhance C2 synapses (data not shown), this prolonged elevation in the spontaneous DSI firing rate may contribute to behavioural changes observed in sensitized animals, such as lowered threshold and onset latency for swimming^{12,13}.

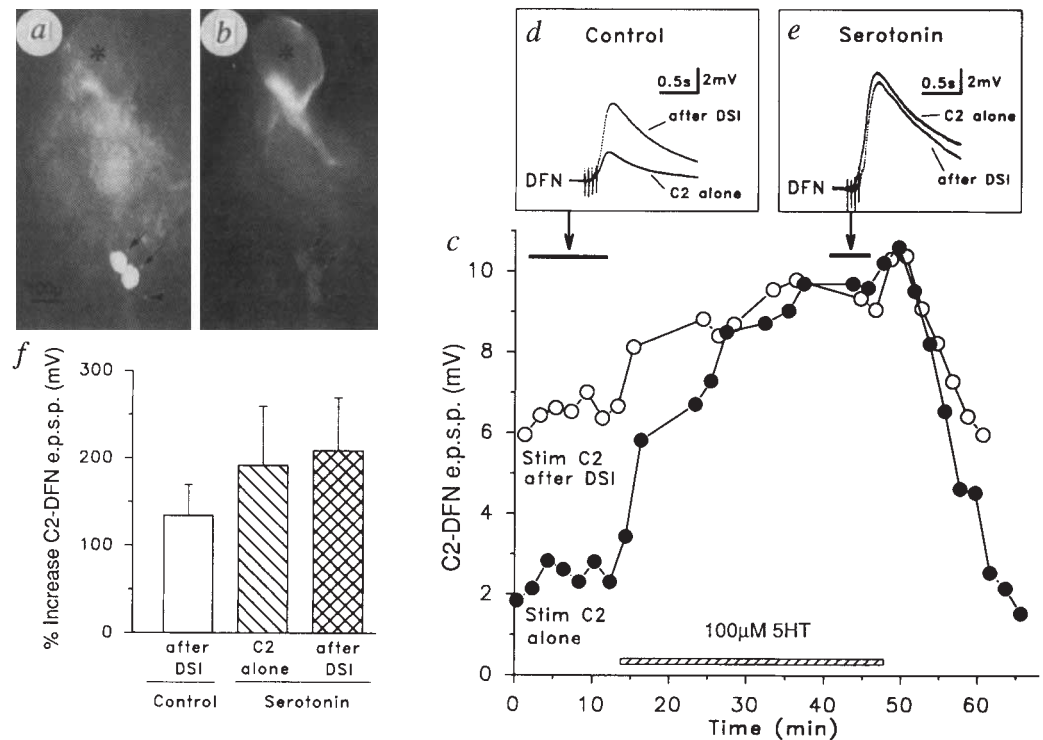
In central pattern generator circuits, all the established sources of neuromodulatory input arise extrinsic to the circuits being modulated^{1,14–16}. The system we have described constitutes an alternative source of neuromodulatory input, intrinsic neuromodulation, where the neuromodulatory neurons are integral components of the circuit being modulated. Although neuromodulation has not previously been shown to be intrinsic to pattern-generating circuits, other components of motor systems have been shown to evoke neuromodulatory effects in addition to their synaptic actions¹⁷. For example, motor neurons, using peptide cotransmitters, modulate neuromuscular properties in a number of systems^{18–20}. Mechanosensory neurons also evoke neuromodulatory effects in reflex circuits^{21,22}. Furthermore, some modulatory neurons, extrinsic to a circuit, receive strong synaptic input on their terminals from that same circuit, making them functional members of the circuit that they are modulating²³. These studies together with our data indicate that intrinsic neuromodulation may be a more general property of circuits than previously appreciated. It provides a mechanism by which circuits can alter their own properties rapidly and dynamically during normal operation. □

FIG. 2 DSI stimulation enhanced the synaptic potentials evoked by C2 inside and outside the CPG. **A**, **a**, C2 stimulation (5 spikes; 20 Hz) evoked a biphasic synaptic potential in a DSI-B neuron (control). Stimulation of another DSI, in this case DSI-C (5 Hz for 10 s; not shown), ending 3 s before the C2 train, increased both the amplitude and duration of the synaptic potential (modulated). **b**, The average C2–DSI i.p.s.p. increased in amplitude from 1.4 ± 0.2 mV to 2.6 ± 0.5 mV (paired Student's *t*-test, $t = 3.37$, $P < 0.01$, $n = 9$) following DSI stimulation (5–7 Hz, 10–20 s). On this and other graphs, error bars represent standard error of the mean. **B**, **a**, C2 stimulation evoked an e.p.s.p. in the DFN (control). DSI stimulation enhanced the amplitude of this e.p.s.p. (modulated). **b**, On average, DSI stimulation (5–7 Hz, 10–20 s) increased the amplitude of the C2–DFN e.p.s.p. from 3.1 ± 0.3 mV to 8.8 ± 1.0 mV (paired Student's *t*-test, $t = 7.40$, $P < 0.001$, $n = 31$). **C**, C2 was stimulated (5 spikes, 20 Hz) before (pre) and in separate trials at 3, 5, 7 and 9 s after cessation of a DSI spike train (5 Hz, 10 s). The direct excitatory DSI–DFN connection can be seen during the DSI train. The amplitude of the C2-evoked e.p.s.p. was enhanced for more than 9 s after cessation of DSI activity. **D**, Average decay of the modulation under conditions identical to those in **C** ($n = 3$). The modulation was maximum at the first point tested (2 s following DSI stimulation). Insets in **A**, **B** and **C** show the cells involved in each experiment. Arrows indicate synapses being modulated by DSI. In all cases, recordings were from isolated brain preparations superfused with saline



at 10 °C and containing increased divalent ion concentrations to limit polysynaptic contributions to the responses. High-divalent-ion saline consisted of (in mM): 285 NaCl, 10 KCl, 25 CaCl₂, 125 MgCl₂, 10 HEPES (pH 7.6), 11 D-glucose. C2 and DSI were stimulated with short current pulses, evoking a single action potential per pulse. DSI modulation of the C2–DFN connection has also been observed in sea water by us (data not shown) and others²⁸.

FIG. 3 Serotonin may mediate the modulatory actions of DSI. *a* and *b*, DSI neurons are immunoreactive for serotonin. *a*, All three ipsilateral DSI neurons were identified physiologically and two were injected with Lucifer yellow (arrows). *b*, The uninjected neuron (arrowhead) and both of the injected neurons (arrows) were recognized by an antibody against serotonin (Incstar, Stillwater, MN), visualized with a secondary antibody tagged with Texas red (Vector Lab). The giant serotonergic cerebral cell C1 (asterisk) displayed serotonin immunoreactivity as expected²⁹. Fixation and antibody procedures were modified from ref. 30. *c*, Serotonin mimics and occludes the modulatory effects of DSI. Brief C2 trains (4 pulses, 20 Hz) were elicited every 60 s to evoke a test e.p.s.p. in DFN; alternate C2 trains were preceded by a DSI train (5 Hz for 10 s; ending 4 s before the C2 train) to induce modulation. In control saline, the C2-DFN e.p.s.p. after DSI stimulation (open circles) was larger than the e.p.s.p. evoked when C2 was stimulated alone (filled circles). Switching the bath solution to 100 μ M serotonin (hatched bar) caused the C2-evoked e.p.s.ps to increase in amplitude, mimicking the effect of DSI stimulation. After 30 min, the e.p.s.p. enhancement caused by serotonin was maximal and the modulatory effect of DSI was occluded. Upon switching back to control saline, the C2-evoked e.p.s.p. returned to its original amplitude and the modulatory actions of DSI reappeared. *d* and *e*, Excerpts from the experiment in *c*, showing averaged compound C2-DFN e.p.s.ps, evoked by C2 stimulation alone and after DSI stimulation, which were taken from the periods



indicated by the black bars, in control saline (*d*) and 100 μ M serotonin (*e*). *f*, Average data from three experiments. DSI produced a 134% enhancement of the C2-DFN e.p.s.p. in control saline. Serotonin mimicked this effect, producing a 191% increase in the C2-DFN e.p.s.p. In serotonin, the ability of DSI to produce further enhancement of the e.p.s.p. was occluded (208% total enhancement). All experiments were done in high-divalent-ion saline and in each case DFN was impaled with two electrodes for independent voltage recording and current injection. The resting potential was held at -70 mV by injecting current into the DFN, countering the direct depolarizing action of serotonin.

The 'blue-on' opponent pathway in primate retina originates from a distinct bistratified ganglion cell type

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COLOUR vision in humans and Old World monkeys begins with the differential activation of three types of cone photoreceptor which are maximally sensitive to short (S), medium (M) and long (L) wavelengths. Signals from the three cone types are relayed to the retinal ganglion cells via cone-specific bipolar cell types¹⁻⁴. Colour-coding ganglion cells fall into two major physiological classes: the red-green opponent cells, which receive antagonistic input from M- and L-sensitive cones, and the blue-yellow opponent cells, which receive input from S-sensitive cones, opposed by combined M- and L-cone input. The neural mechanisms producing colour opponency are not understood. It has been assumed that both kinds of opponent signals are transmitted to the lateral geniculate nucleus by one type of ganglion cell, the midget cell^{5,6}. We now report that a distinct non-midget ganglion cell type, the small bistratified cell, corresponds to the physiological type that receives

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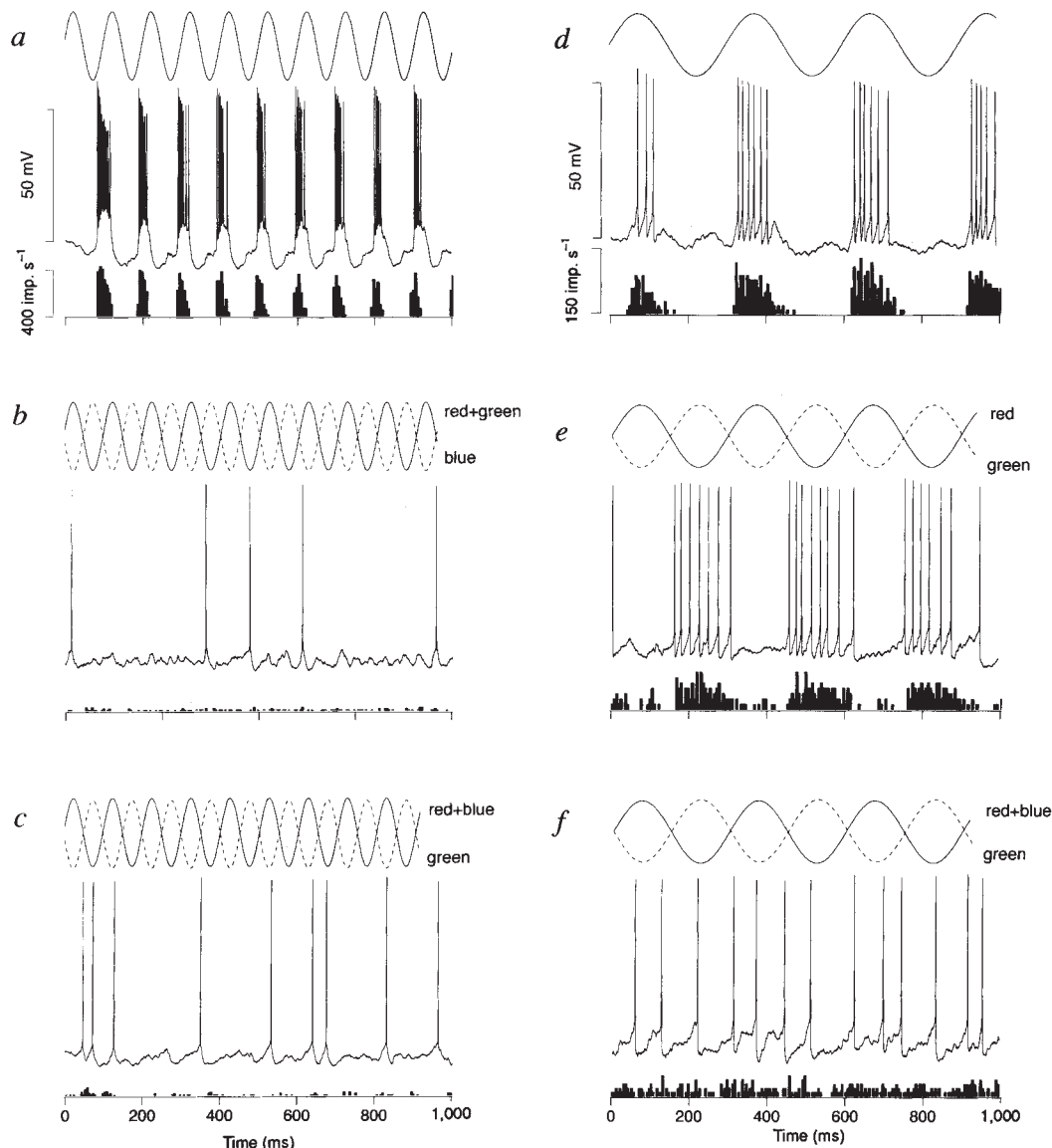
excitatory input from S cones, the 'blue-on' cell. Our results thus demonstrate an anatomically distinct pathway that conveys S-cone signals to the brain. The morphology of the blue-on cell also suggests a novel hypothesis for the retinal circuitry underlying the blue-yellow opponent response.

Blue-on cells were identified physiologically by intracellular recording in an *in vitro* preparation of the macaque retina. Dur-

ing recording, tracer was injected into the cell so that its morphology could be determined. Because previous intracellular studies in primates on the intact eye^{7,8} or isolated eye cup^{7,9} have been only partially successful, we used a different approach. We isolated the retina, together with the pigment epithelium and choroid, and mounted this preparation flat in a recording chamber. Complete removal of the vitreous promoted survival

FIG. 1 Intracellular responses (impulses (imp.) per s) of morphologically identified parasol and midget ganglion cells of *Macaca nemestrina* in an isolated retina-choroid preparation. **a**, Strong visual response of a cell, identified morphologically as an outer parasol cell, to the output of red and green light-emitting diodes (LEDs) modulated in phase at 10 Hz. The cell gave a phasic off-response and hyperpolarized in response to luminance increment. Top trace, stimulus composition (modulation contrast is 100% in all figures); middle trace, intracellular record; bottom, a peristimulus time histogram averaged over 20 stimulus presentations. **b**, When the output of the blue diode was adjusted to equal luminance and modulated 180° out of phase with modulation of the red and green diodes in phase (blue/yellow chromatic flicker), no response was elicited from the cell. **c**, Similarly, stimuli modulated along a tritanopic confusion line, where only S cones are modulated, evoked no response. **d-f**, Visual response of a cell identified morphologically as an inner midget cell. Luminance modulation at 3.3 Hz elicited an on-response (**d**); chromatic modulation between red and green elicited a green-on, red-off response (**e**). No midget cells gave any modulated response to the S-cone isolating stimulus (**f**).

METHODS. Eyes (*M. nemestrina*, *Macaca fascicularis* or *Macaca mulatta*) were obtained from the tissue programme of the Washington Regional Primate Research Center. The retinæ were prepared by draining the vitreous from the eye cup and dissecting the retina, pigment epithelium and choroid intact in oxygenated Ames medium. The retina-choroid was mounted in a superfusion chamber on the stage of a light microscope and kept at $36 \pm 1^\circ\text{C}$. Ganglion cell bodies were visualized by epifluorescence after staining with acridine orange. Intracellular penetrations were made under direct microscopic control and cells were recorded in conventional current clamp mode. Red, green and blue LEDs were used to deliver chromatic stimuli via a small optical bench mounted on the camera port of the microscope. Output of the LEDs was sinusoidally modulated¹¹. The red, green and blue LEDs have dominant wavelengths of about 638, 554 and 470 nm, respectively. The irradiance in the plane of the retina was measured using a Gamma Scientific spectral radiometer. We estimated retinal illuminance in these experiments to have been ~ 100 trolands. To produce the S-cone isolating



stimulus, human observers used flicker photometry to estimate the relative luminances of the red, green and blue diodes on white paper set on the microscope stage in the plane of the retina. These relative troland values were then corrected for the absence of macular pigmentation (all recordings were done in retinal periphery), lens and ocular media²⁶. The relative intensities predicted for an S-cone isolating stimulus were then calculated from the cone fundamentals²⁷, checked and adjusted as described elsewhere (T. Yeh, B.B.L. and J. Kremers, manuscript in preparation). Our measurements showed the retinæ to be in good physiological condition. MC cells responded down to 1–2% luminance contrast, and the S-cone cells to 5–10% S-cone modulation. These are comparable to sensitivities found *in vivo*. Also, MC-cell flicker photometric spectral sensitivities were close to the prediction, indicating that cone weightings had not been disturbed during dissection of the retina. The dendritic morphology of the recorded cells was visualized initially through the microscope by intracellular injection of the fluorescent dye pyranine during recording, and preserved for later analysis by injection of Neurobiotin (Vector; Burlingame, CA) and subsequent horseradish peroxidase histochemistry²⁸.

of the retina and we commonly obtained vigorous light responses for many hours. A crucial advantage of the *in vitro* preparation was that the retina was mounted on the stage of a light microscope so that we were able to target identified ganglion cell types visually for recording¹⁰. Retinae were vitally stained with a fluorescent dye, acridine orange, which clearly revealed the cell bodies of the two major ganglion cell classes, the large-bodied parasol cells and the small-bodied midget cells, whose axons project to the magnocellular (MC) and parvocellular (PC) layers of the lateral geniculate nucleus (LGN), respectively. Light stimuli, delivered as sinusoidal flicker from red, green and blue light-emitting diodes¹¹, were projected onto the retinal surface via the camera port of the microscope. Episcopic illumination was required to visualize and penetrate individual cell bodies, but this did not appreciably bleach the cone photopigments, as vigorous light responses could be recorded immediately after cell penetration.

We first recorded intracellularly from the two major ganglion cell classes whose axons project to the LGN, and investigated whether these cells showed any evidence of S-cone input. Morphologically identified parasol cells ($n=25$) corresponded, as predicted from previous results^{5,6}, to phasic, non-opponent cells (MC cells). As expected¹², parasol cells whose dendrites stratified in the inner portion of the inner plexiform layer (IPL) gave 'on-centre' responses to luminance, and those that stratified in the outer IPL gave 'off-centre' responses. Phasic parasol cells gave no modulated responses to isoluminant chromatic stimuli optimized to reveal input from S-cones, consistent with previous conclusions that these cells receive little or no S-cone input¹³. Figure 1 shows responses of an on-centre phasic cell. Although the cell responded strongly to luminance modulation (Fig. 1*a*), there was no response to blue-yellow chromatic flicker (Fig. 1*b*) or to a stimulus modulated along a tritanopic confusion line (Fig. 1*c*), a condition in which only S-cones are modulated. Recordings from morphologically identified midget ganglion cells¹⁴ in the retinal periphery revealed, as expected, responses to both luminance modulation and red-green chromatic modulation⁴. However, like the parasol cells, these midget ganglion cells ($n=18$) showed no evidence of input from S cones. Figure 1*d-f* illustrates the response of a green-on, red-off colour opponent cell identified as an inner midget ganglion cell. This

cell showed a clear opponent response to red and green set at equal luminance and modulated 180° out of phase (Fig. 1e). S-cone isolating stimuli (Fig. 1f) did not elicit a response.

Given the paucity of S cones in the retina, it is conceivable that S-cone signals are transmitted to the LGN by only a small percentage of midget cells and our sample could have been too small to discover them. An alternative possibility is that a third ganglion cell type could convey S-cone signals to the LGN. We tested this by targeting a recently identified primate ganglion cell type, the small bistratified cell, whose axon projects to the parvocellular geniculate layers¹⁵. We were able to target the small bistratified cell for intracellular injection and recording *in vitro* by its characteristic cell body size and spatial density¹⁶.

All small bistratified cells recorded ($n=23$) received strong excitatory input from S cones and resembled the blue-on cell that has been described physiologically¹⁷. All small bistratified cells gave a strong response to the S-cone isolating stimulus (Fig. 2), such that membrane depolarization and associated action potentials were always in phase with the S-cone modulation. The small bistratified cells also gave a sustained on-response to blue luminance modulation (Fig. 3*a*) and a clear off-response to yellow luminance modulation; isoluminant blue-yellow modulation elicited a strong blue-on response. Although it has been suggested that more than one cell class may receive excitatory S-cone input (F. M. De Monasterio and A. Mariani, personal communication), among the various non-midget, non-parasol ganglion cell types from which we have recorded, only the small bistratified cells have shown evidence of excitatory input from S-cones. In particular, we have found no cells that have both parasol-like morphology⁷ and excitatory S-cone input.

The distinctive morphology of the small bistratified cell suggests a specific neural circuitry that may give rise to blue-yellow colour opponency. The larger, inner dendritic field stratifies at precisely the same depth as the axon terminals of the blue-cone bipolar cells^{2,16}, making it likely that the blue-cone bipolar cell and the small bistratified cells form a synaptic pathway for excitatory S-cone signals in the retina. It seems probable that a second, hyperpolarizing bipolar cell type conveys M- and L-cone input directly to the outer dendritic tree. This arrangement would be consistent with the strong off-response to yellow luminance modulation shown in Fig. 3*b* and with the receptive field

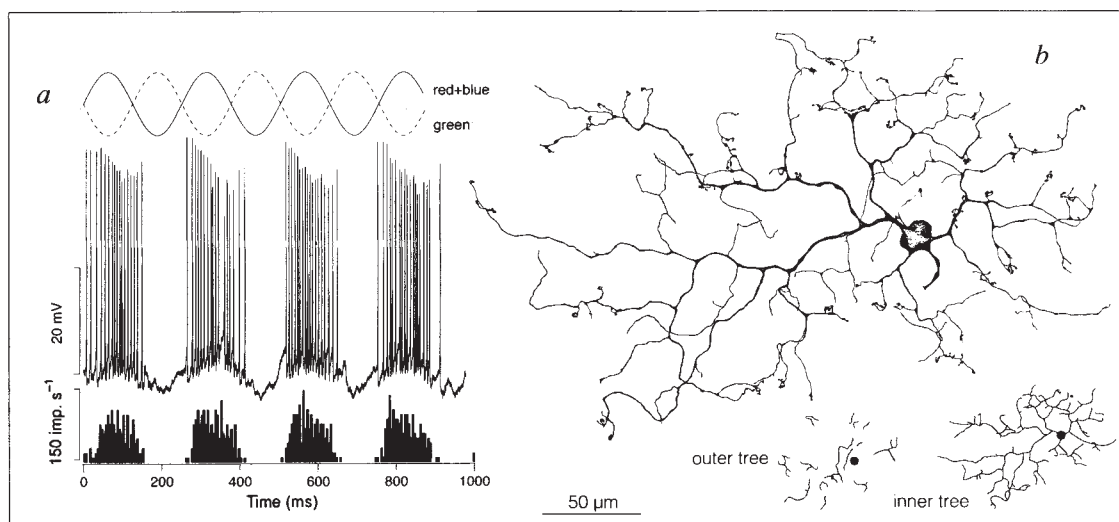


FIG. 2 Physiological and morphological identification of the blue-on ganglion cell type. *a*, The small bistratified cell gave a vigorous and sustained on-response to the same S-cone isolating stimulus that elicited null responses from the red-green opponent and phasic non-opponent cells shown in Fig. 1. Stimulus presentation at 4 Hz; all conventions as in Fig. 1. *b*, Dendritic morphology of cell whose intracellular

recording is shown in a displayed the pattern of bistratification and morphological features of all small bistratified cells¹⁶. The two tiers of the dendritic tree are traced separately (shaded insets) and illustrate that the inner tree forms a larger, more densely branched field than the outer dendrites.

FIG. 3 Small bistratified cells also gave characteristic blue-on, yellow-off responses. *a*, Intracellular recording of response to 4 Hz luminance modulation of blue LED. The cell showed a sustained on-response with hyperpolarization of membrane potential to luminance decrement. *b*, Off-response of the same cell to luminance modulation of a yellow light (red and green LEDs modulated in phase). The cell was hyperpolarized by a yellow luminance increment and gave a burst of spikes with luminance decrement. *c*, Response of the same cell to blue-yellow isoluminant chromatic modulation. Blue LED sine wave (solid line) is 180° out of phase with the in-phase modulation of the red and green LEDs (dotted line). *d*, Dendritic morphology of the small bistratified ganglion cell whose light response is shown in *a*, *b* and *c*. This cell was located at a lesser retinal eccentricity than the cell shown in Fig. 2 and consequently had a smaller overall dendritic field size¹⁶.

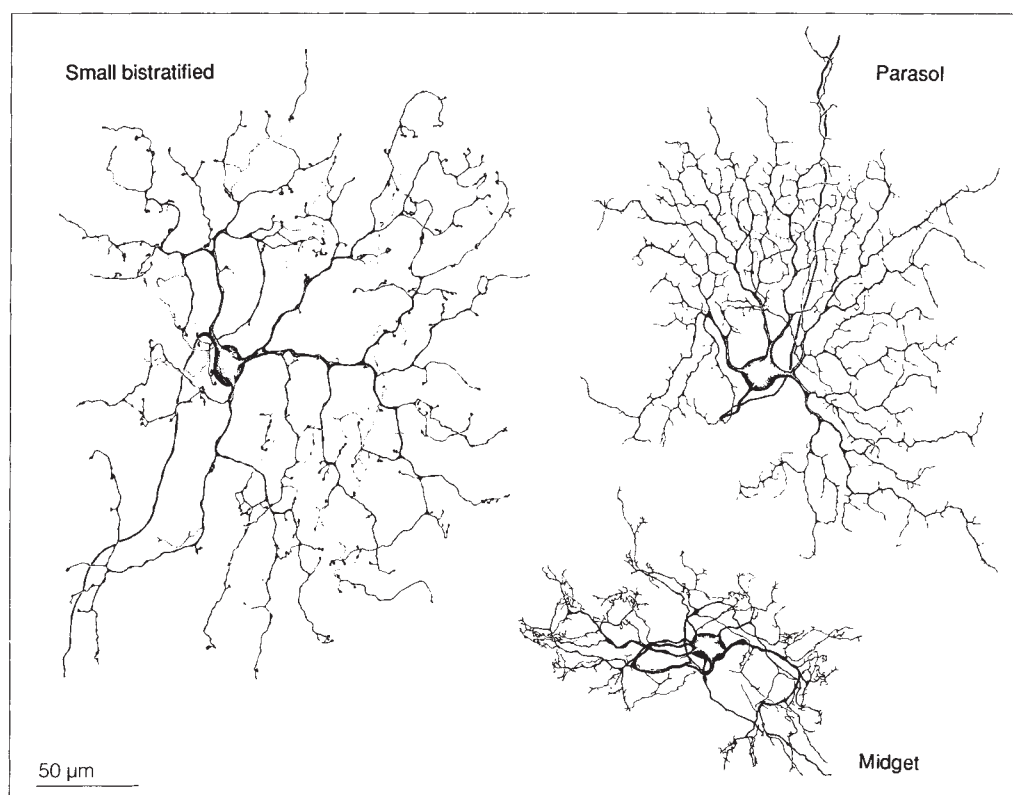
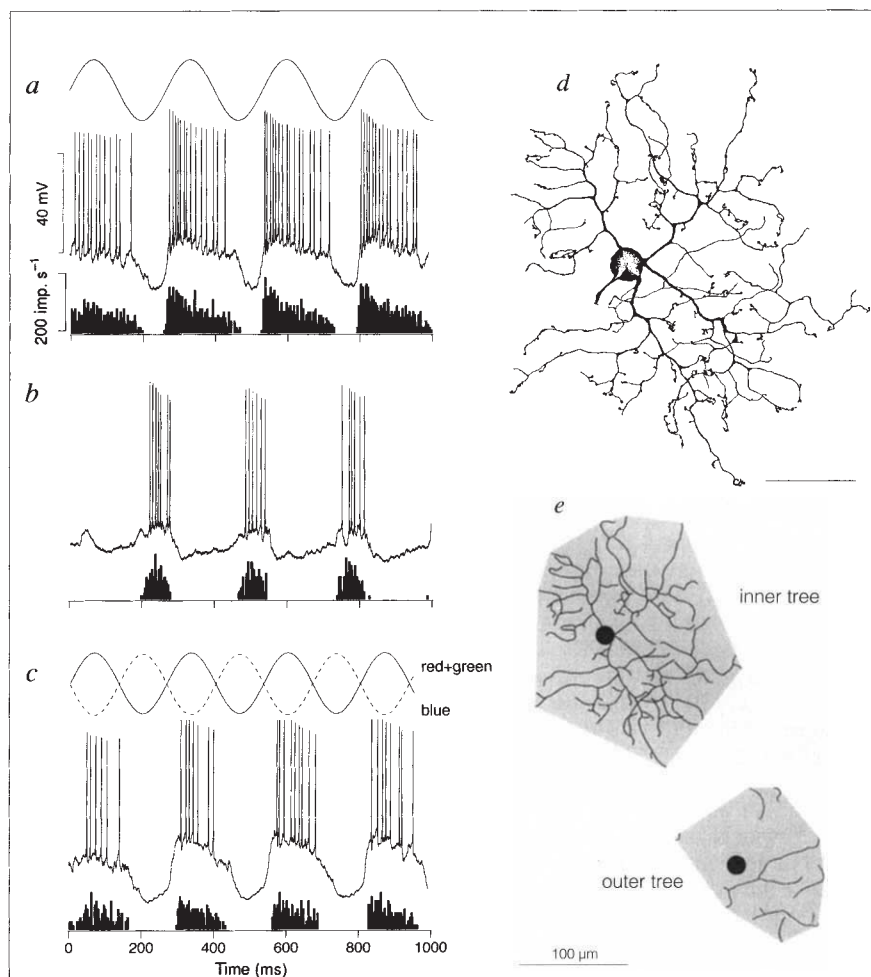


FIG. 4 Camera lucida tracings comparing the dendritic morphology of macaque ganglion cell types from the retinal periphery whose axons project to the dorsal lateral geniculate nucleus. The parasol cells give phasic, non-opponent on- or off-centre responses and project to the magnocellular LGN. The midget ganglion cells give red-green on- or off-centre opponent responses and project to the parvocellular LGN. The small bistratified ganglion cells give blue-yellow opponent responses of the blue-on type and also project to the parvocellular LGN. The blue-on, small bistratified ganglion cells have a dendritic field size similar to that of the non-opponent parasol cells, but differ from the parasol cells in the detailed shape of the dendritic tree.

structure of most blue-on cells, which are organized into spatially coextensive on and off regions^{18,19}.

Our results demonstrate an anatomical dichotomy in the primate parvocellular pathway which corresponds to the physiological dichotomy between the M, L- and S-cone systems. Midget ganglion cells convey M, L-cone opponent signals and the small bistratified cells convey S-cone excitatory signals (Fig. 4). It is not known whether there are other parallel pathways to the parvocellular layers. A blue-off cell type has been described^{18,20} and the possibility that ganglion cell types other than the midget cells convey M, L-cone opponent signals has been suggested¹⁶, but we have not encountered such cells.

Most mammals possess an S-cone system, but the existence of distinct M and L cones, and their use for trichromatic vision, seems to be a relatively recent evolutionary event²¹ which reflects a specialized adaptation in certain primates^{22,23}. The identification of separate ganglion cell types devoted to S-cone and M, L-cone signals is consistent with this picture of the evolution of primate colour vision. Moreover, our result supports a previous suggestion that the blue-on pathway may be kept anatomically segregated at the level of the LGN²⁴ and primary visual cortex²⁵, but its significance for further understanding the central pathways for colour-coding in primates remains largely to be explored. □

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Dynamics of synaptic vesicle fusion and membrane retrieval in synaptic terminals

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COMMUNICATION among neurons occurs at specialized synaptic junctions, where neurotransmitter is released via calcium-dependent exocytosis from the synaptic terminal of the presynaptic cell onto the postsynaptic target neuron. Here we exploit the unique properties of giant synaptic terminals^{1–4} of bipolar neurons from goldfish retina to establish the kinetics and calcium-dependence of exocytosis, and the characteristics of membrane retrieval following secretion in presynaptic terminals. Simultaneous patch-clamp, calcium-indicator dye and time-resolved capacitance measurements reveal that activation of calcium current drives secretion at a rapid rate of about 10,000 vesicles per s and the calcium level necessary to drive secretion is locally greater than 50 μ M. Two components of membrane retrieval were observed following secretory stimulation. After strong stimulation, capacitance returned to rest with a time constant of about 30 s, but after weaker stimuli recovery was much faster, with a time constant of about 2 s. Secretion in a vertebrate central nervous system neuron was thus found to differ substantially from that in other secretory cells in its rapid rate of vesicle fusion, requirement for high levels of intracellular calcium, and the high speed and completeness of membrane retrieval. These distinctive features reflect the specialization of neuronal synaptic terminals for rapid and focally directed release of neurotransmitter.

Giant synaptic terminals of goldfish (*Carassius auratus*) retinal bipolar neurons are about 10 μ m in diameter (Fig. 1a) and contain approximately 60 ribbon-type output sites⁵, each with

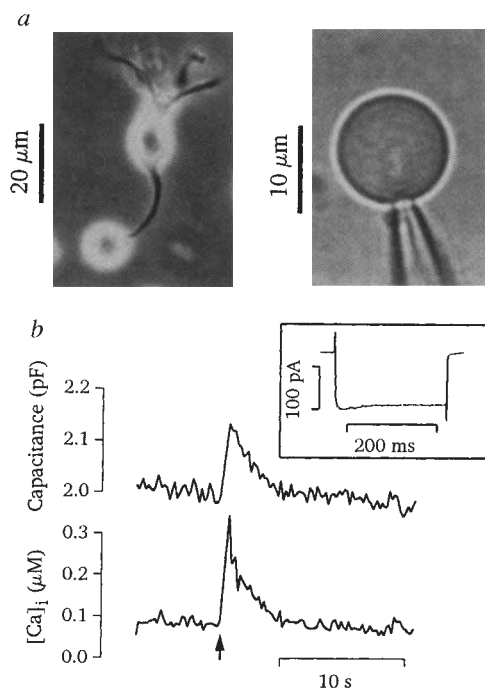
many small clear-core vesicles (~50 nm in diameter) tethered to the ribbon. Although the vesicles are small, the summed increase in membrane area at all of the output sites during secretion is sufficient to produce a detectable increase in capacitance (Fig. 1b). Activation of Ca^{2+} current (see inset, Fig. 1b) elicited an increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$), accompanied by a rapid increase in capacitance, which returned to baseline within a few seconds. The capacitance response can be readily interpreted in terms of the dynamics of synaptic vesicle release and recycling. The rapid jump in capacitance in response to depolarization corresponds to the fusion (exocytosis) of a readily releasable pool of vesicles, such as those tethered to synaptic ribbons at release sites. The restoration of capacitance to resting level corresponds to membrane retrieval (endocytosis) and vesicle recycling^{6,7}. The amplitude of the jump in capacitance after a single 250-ms depolarization averaged 154 ± 36 fF ($n = 27$), corresponding to the fusion of ~2,000 vesicles, or ~30 vesicles at each of the ~60 ribbon-type output sites.

To determine the rate of vesicle fusion, the duration of depolarization was varied (Fig. 2a). For pulse durations up to 180 ms, the capacitance response increased linearly with pulse duration at a rate of 730 fF s⁻¹, or ~10,000 vesicles s⁻¹. This rapidly releasable pool of vesicles was limited in size, however, because increasing the pulse duration beyond 180 ms caused no further increase in the capacitance jump. Figure 2b shows the dependence of the capacitance jump on the amplitude of depolarization. At voltages more negative than -20 mV, there was little change in capacitance, although Ca^{2+} current and increases in $[\text{Ca}^{2+}]_i$ were observed in this range of membrane potential (Fig. 2c). With further depolarization, Ca^{2+} current and the capacitance response increased, reaching a peak at 0 mV. At more positive membrane voltages, the capacitance response declined progressively until no change could be detected at potentials more positive than +50 mV. A similar relationship was observed for $[\text{Ca}^{2+}]_i$ (Fig. 2c), which suggests that the capacitance change was due to Ca^{2+} -dependent exocytosis.

To examine the calcium dependence of the capacitance response, exogenous calcium buffers (BAPTA and EGTA) were

FIG. 1 Changes in intracellular calcium concentration and membrane capacitance in an isolated synaptic terminal of a retinal bipolar neuron. **a**, Phase-contrast picture (left) shows an intact goldfish retinal bipolar neuron, with dendrites, cell body, axon and a giant synaptic terminal. The cell morphology is identical to type Mb1 bipolar cells in Golgi-stained goldfish retina^{1,2}. The bright-field view on the right shows an isolated synaptic terminal with a whole-cell patch pipette used to control membrane voltage, record membrane current and load the terminal with calcium-indicator dye (Fura-2 or Fura-2/AM). **b**, Activation of Ca^{2+} current increased $[\text{Ca}^{2+}]_i$ and capacitance of an isolated synaptic terminal. At the arrow, a 250-ms depolarization from -60 mV to 0 mV was given, producing the inward Ca^{2+} current shown in the inset. Both Ca^{2+} accumulation⁴ and neurotransmitter (glutamate) release¹⁴ have been shown to depend on this slowly inactivating, dihydropyridine-sensitive Ca^{2+} current. Pipette solution contained 0.5 mM BAPTA and 0.1 mM Fura-2. Because of the conductance increase during activation of Ca^{2+} channels, it was not possible to record capacitance during the depolarizing pulse¹⁵. However, the capacitance jump was largest at the first sample after the depolarization, suggesting that all of the secretion actually occurred during the voltage-clamp pulse. The capacitance response of isolated terminals frequently declined with time during whole-terminal recordings and disappeared entirely within ~ 10 min after break-in. The Ca^{2+} current and the associated changes in $[\text{Ca}^{2+}]_i$ typically showed little change on this timescale, so the decline in capacitance response cannot be attributed to reduced Ca^{2+} influx. Such washout of the secretory response has been reported in other secretory cells¹³ and may be due to loss of a soluble factor during dialysis via the whole-cell patch pipette.

METHODS. Bipolar neurons were obtained by mechanical trituration after papain digestion of goldfish retina as described^{3,4}. Isolated terminals were obtained by mechanically cleaving the connecting axon of an intact cell or by visually selecting spontaneously isolated terminals, whose identity was confirmed on the basis of their distinctive electrophysiological profile (large, slowly inactivating Ca^{2+} current; absence of voltage-gated Na^+ current; absence of transient Ca^{2+} current). All recordings were made at 20 – 25°C from acutely isolated terminals within 2–5 h of dissociation. The extracellular saline consisted of (in mM): NaCl, 115; KCl, 2.6; MgCl_2 , 1.6; CaCl_2 , 2.5; HEPES–NaOH, 10; pH 7.3. Patch pipettes were coated with dental wax to reduce stray capacitance and were filled with an intracellular solution containing (in mM): Cs-gluconate, 130; TEA-Cl, 10; MgCl_2 , 2; $\text{Na}_2\text{-ATP}$, 2; GTP, 0.5; HEPES–CsOH, 10–25; pH 7.2. Varying amounts of EGTA or BAPTA were



added to the pipette solution as indicated in descriptions of individual experiments. Calcium indicators Fura-2 or Fura-2/AM (Magenta; Molecular Probes) were added at 0.1 mM when indicated. Dual-wave-length fluorescence excitation, photometric measurement of emitted light and calibrated calculation of free $[\text{Ca}^{2+}]_i$ were as described previously⁴. Membrane capacitance was measured with a sinusoidal voltage-clamp command and a two-phase lock-in amplifier^{15–17}, or with the automatic capacitance compensation feature of the EPC-9 patch-clamp amplifier¹⁸. Both methods gave equivalent results. Capacitance measurements have been used to study secretion in a variety of cell types, including oocytes¹⁶, chromaffin cells¹⁷, mast cells¹⁹, neuroendocrine nerve terminals^{20,21} and pituitary melanotrophs^{22,23}.

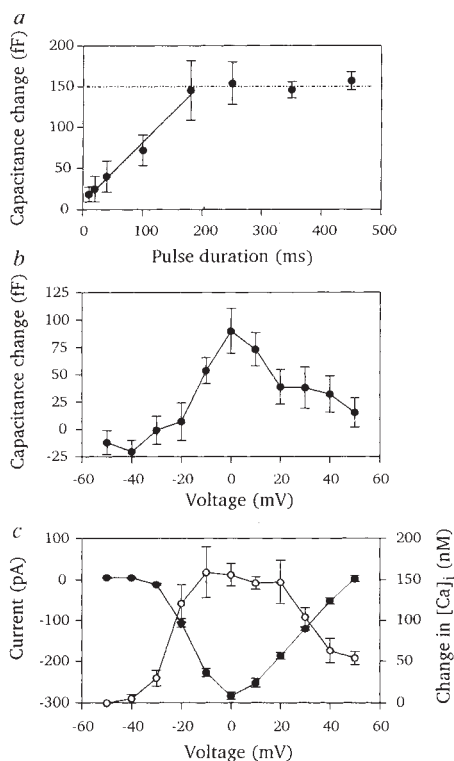


FIG. 2 Properties of the rapid jump in capacitance elicited by brief depolarization of isolated synaptic terminals. **a**, The relation between duration of depolarization (to 0 mV) and size of the capacitance jump. Data points show averages ($\pm$ s.e.m.) from 5–27 terminals. For pulse durations <100 ms, responses to 5–25 pulses were averaged for each terminal. The solid line fitted to the results for durations <200 ms had a slope of 730 fF s^{-1} , corresponding to about $10,000$ vesicles s^{-1} , or ~ 0.2 vesicles per ms per site, assuming 60 ribbon-type release sites in the terminal⁵. This rapid initial rate of vesicle fusion is comparable to that at the neuromuscular junction, where about 150 vesicles²⁴ are released from ~ 300 release sites²⁵ during an action potential; taking the duration of the action potential to be 1 ms, this corresponds to 0.5 vesicles per ms per site. The dashed line indicates the plateau level of 150 fF, attained at durations >200 ms. The data could also be fitted, although less well, with a single exponential time constant of 124 ms, which corresponds to an initial secretion rate of $\sim 16,000$ vesicles s^{-1} (see ref. 23), assuming a releasable pool of $2,000$ vesicles. **b**, Voltage-dependence of the capacitance jump elicited by single depolarizing pulses. The pulse duration was fixed at 100 ms. Data points are means $\pm$ s.e.m. from 4–16 terminals; for each terminal, responses to 5–15 pulses were averaged. The capacitance–voltage relation is similar to the voltage-dependence of neurotransmitter release from bipolar-cell synaptic terminals, monitored by transmitter-activated currents²⁶. This similarity suggests that the capacitance changes reflect release of neurotransmitter. **c**, Voltage-dependence of calcium current (filled circles) and the associated change in $[\text{Ca}^{2+}]_i$ (open circles) for the same set of terminals as **b**. The intracellular solution included 0.4 mM BAPTA and 0.1 mM Fura-2. The overall shape of the relation between voltage and $[\text{Ca}^{2+}]_i$ was similar to that between voltage and capacitance. However, both the rise and fall in capacitance with increasing depolarization (Fig. 2b) were more abrupt than the accompanying changes in $[\text{Ca}^{2+}]_i$. Such sharpening of the relationship between capacitance and voltage might arise, for instance, if secretion depends on $[\text{Ca}^{2+}]_i$ raised to a power²².

added via the patch pipette. With 0.5 mM BAPTA, the capacitance change during a train of depolarizing pulses showed a rapid jump after the first pulse, followed by a slow rise to a plateau of elevated capacitance (Fig. 3a; open circles). But with 5 mM BAPTA, the capacitance change was delayed (Fig. 3a; filled circles), which is reminiscent of the inhibition by BAPTA of synaptic transmission at the squid giant synapse⁸. This provides further evidence that the capacitance change in bipolar-cell terminals reflects calcium-dependent exocytosis. The much slower Ca^{2+} buffer EGTA has little effect, even at 10 mM (data not shown). The fact that high concentrations of a fast buffer were required to affect the capacitance response suggests that secretion is driven by the high levels of Ca^{2+} expected near Ca^{2+} channels⁹⁻¹¹, rather than by the smaller and slower changes in

bulk $[\text{Ca}^{2+}]_i$ measured in our Fura-2 experiments.

Application of the calcium ionophore ionomycin also increased both $[\text{Ca}^{2+}]_i$ and capacitance (Fig. 3b), but the response was slower than that driven by depolarization, averaging $38 \pm 5 \text{ fF s}^{-1}$ ($n=8$), or 480 vesicles s^{-1} , compared with $\sim 10,000$ vesicles s^{-1} for secretion elicited by Ca^{2+} current. This may partly reflect the strategic localization of Ca^{2+} channels at active zones and partly the more synchronous activation of secretion throughout the terminal that results from the abrupt activation of Ca^{2+} current by depolarizing voltage-clamp pulses. Fura-2 was saturated at the level of $[\text{Ca}^{2+}]_i$ achieved during ionomycin application, so we used the less sensitive Ca^{2+} indicator Fura-2 (ref. 12) to monitor the increase in $[\text{Ca}^{2+}]_i$ during ionomycin application (Fig. 3b; bottom trace). Ionomycin

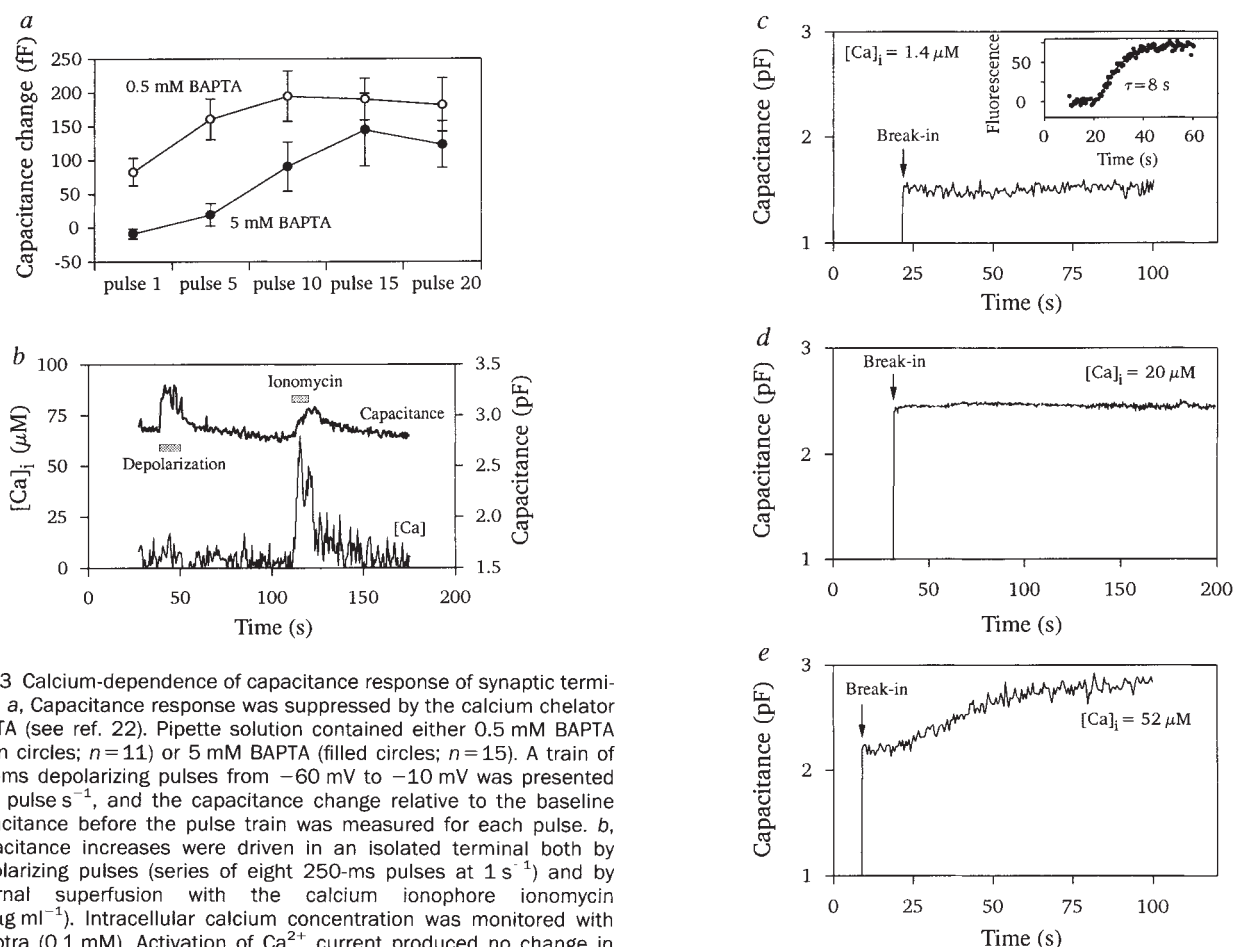


FIG. 3 Calcium-dependence of capacitance response of synaptic terminals. **a**, Capacitance response was suppressed by the calcium chelator BAPTA (see ref. 22). Pipette solution contained either 0.5 mM BAPTA (open circles; $n=11$) or 5 mM BAPTA (filled circles; $n=15$). A train of 250-ms depolarizing pulses from -60 mV to -10 mV was presented at 1 pulse s^{-1} , and the capacitance change relative to the baseline capacitance before the pulse train was measured for each pulse. **b**, Capacitance increases were driven in an isolated terminal both by depolarizing pulses (series of eight 250-ms pulses at 1 s^{-1}) and by external superfusion with the calcium ionophore ionomycin ($10 \mu\text{g ml}^{-1}$). Intracellular calcium concentration was monitored with fura-2 (0.1 mM). Activation of Ca^{2+} current produced no change in $[\text{Ca}^{2+}]_i$ detected by Fura-2, as expected from the high dissociation constant of Fura-2 for Ca ($\sim 50 \mu\text{M}$)¹² relative to the spatially averaged level of $[\text{Ca}^{2+}]_i$ achieved during depolarization ($<1 \mu\text{M}$). **c**, Capacitance of a synaptic terminal did not change during dialysis with pipette solution containing $[\text{Ca}^{2+}]_i$ $1.4 \mu\text{M}$ (average rate of change of capacitance, $-0.8 \pm 0.7 \text{ fF s}^{-1}$; $n=12$). The inset shows the exponential rise (time constant $\tau=8 \text{ s}$) of fluorescence following break-in as Fura-2 entered the terminal, confirming that dialysis was rapid. The $[\text{Ca}^{2+}]_i$ indicated by Fura-2 was $>1 \mu\text{M}$. However, the terminal was capable of secretion, because capacitance increased when the Ca^{2+} current was activated (data not shown). **d**, Dialysis of a terminal with pipette solution containing $[\text{Ca}^{2+}]_i$ $20 \mu\text{M}$ (average rate of change of capacitance, $0.4 \pm 0.1 \text{ fF s}^{-1}$; $n=6$). The time constant of dialysis was 16 s. **e**, Dialysis of a terminal with pipette solution containing $[\text{Ca}^{2+}]_i$ $52 \mu\text{M}$. The time constant of rise of Fura-2 fluorescence in this experiment was 12 s. The level of free Ca^{2+} required to trigger secretion was 50-fold higher than in chromaffin cells¹³. This difference in Ca^{2+} sensitivity is like that reported in hippocampal synaptosomes²⁷ for release of glutamate, which is focally released at active zones and requires high

levels of Ca^{2+} compared with release of neuropeptides and catecholamines, which are diffusely released and require lower levels of Ca^{2+} . **METHODS.** In experiments in which Ca^{2+} was buffered to high levels in the pipette solution, the lower-affinity calcium chelators HEEDTA (*N*-hydroxyethylethylenediaminetriacetic acid) or NTA (nitrilotriacetic acid) were used¹³, and chloride was substituted for gluconate to avoid Ca precipitation. For the calculated $1.4 \mu\text{M}$ Ca solution, the pipette contained 10 mM HEEDTA (neutralized with CsOH) and 3 mM CaCl_2 . In some experiments, an alternative $1.4 \mu\text{M}$ Ca solution was used which contained 9 mM Ca-EGTA/1 mM EGTA¹³. Free $[\text{Ca}^{2+}]$ measured with a Ca-sensitive electrode was $1.2 \mu\text{M}$ in the HEEDTA solution and $2.0 \mu\text{M}$ in the EGTA solution; Fura-2 measurements during experiments confirmed free $[\text{Ca}^{2+}]_i > 1 \mu\text{M}$. The $20 \mu\text{M}$ Ca solution contained 10 mM NTA + 1.45 mM CaCl_2 . The calcium level was confirmed by including 0.1 mM Fura-2 in the pipette (data not shown). The solution buffered to $52 \mu\text{M}$ Ca^{2+} contained 10 mM NTA and 3 mM CaCl_2 . Free $[\text{Ca}^{2+}]$ in this solution was estimated at $68 \mu\text{M}$ with a Ca-sensitive electrode.

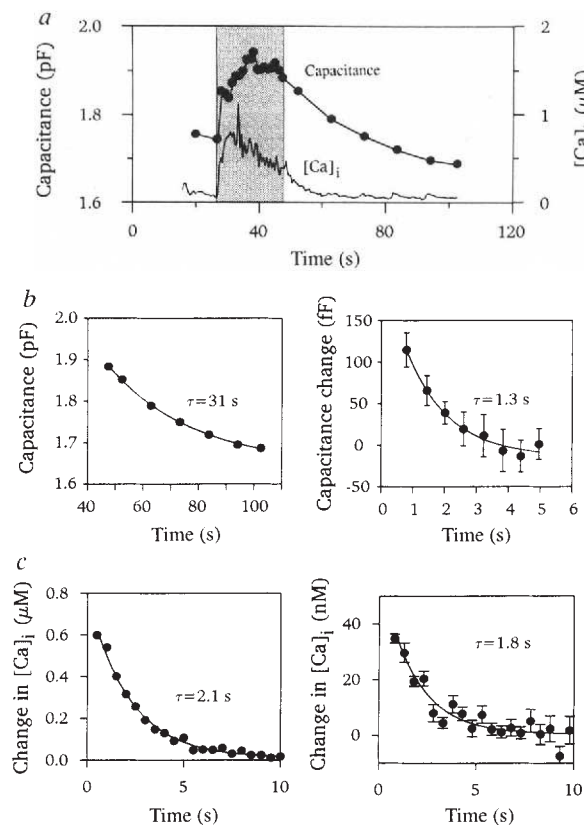


FIG. 4 The rate of membrane retrieval depends on the strength of stimulation. **a**, The increase in capacitance and in $[Ca^{2+}]_i$ during a train of 250-ms pulses from -60 mV to -10 mV given at 1 s $^{-1}$ (shaded region). Outside the shaded region, capacitance was monitored with single brief depolarizing pulses given at 8-s intervals on average. During the series of pulses, $[Ca^{2+}]_i$ within the terminal remained elevated, and the capacitance increased (shaded region). The capacitance change consisted of three phases: a rapid jump evident after the first pulse, a sustained elevation during maintained stimulation, and a retrieval process (endocytosis). The sustained elevation in capacitance during repetitive pulses represents a balance among competing factors, including depletion of releasable vesicles, restoration of the releasable pool, Ca-dependent inactivation of Ca^{2+} current, and membrane retrieval. After the series of pulses, $[Ca^{2+}]_i$ returned rapidly to baseline; the capacitance also returned to baseline, reflecting retrieval of membrane. The retrieval process was slower than the return to resting $[Ca^{2+}]_i$. In this experiment 1 mM EGTA and 0.1 mM Fura-2 were added to the pipette. **b**, Rate of recovery of the capacitance after a series of depolarizing pulses (left) or after a single 250-ms pulse (right). The single-pulse data show the average response to 15 pulses. The solid lines in each panel show exponential decays with the indicated time constants. In seven cells in which both strong and weak stimuli were given, the recovery time constant averaged 23 ± 7 s after strong stimuli and 2 ± 0.5 s after weak stimuli. Candidates for the retrieval mechanisms underlying the two rates might be clathrin-dependent²⁸ and clathrin-independent²⁹ pathways. Alternatively, there may be a single retrieval process whose rate is regulated in response to the degree of stimulation, as might occur, for instance, if the retrieval rate depends on the average level of $[Ca^{2+}]_i$ in the terminal. The slower rate after strong stimulation was similar to the retrieval rate observed using dye-labelling in synapses of cultured hippocampal neurons following prolonged potassium depolarization³⁰. **c**, Recovery of $[Ca^{2+}]_i$ after a series of 250-ms pulses or after a single pulse. The single-pulse data show averages of seven pulses given at one per 20 s. Pipette solution contained 0.5 mM BAPTA and 0.1 mM Fura-2 for **b** and **c**.

increased $[Ca^{2+}]_i$ to a peak of about 50 μM, which we take to be a lower bound for the level of $[Ca^{2+}]_i$ achieved at release sites during depolarization.

In response to depolarizing stimuli, spatially averaged $[Ca^{2+}]_i$ did not typically exceed 1 μM as measured with Fura-2, even during long trains of pulses (see, for example, Fig. 4a). However, local $[Ca^{2+}]_i$ at synaptic release sites is probably substantially higher than the bulk $[Ca^{2+}]_i$ averaged across the terminal^{9,11,13}. To verify directly the $[Ca^{2+}]_i$ level needed to elicit secretion, synaptic terminals were dialysed with pipette solution containing elevated $[Ca^{2+}]_i$. Figure 3c shows the response of a terminal to dialysis with $[Ca^{2+}]_i$ buffered at 1.4 μM (ten times the resting $[Ca^{2+}]_i$). In chromaffin cells, this is a level of $[Ca^{2+}]_i$ sufficient to drive secretion maximally¹³, but in bipolar-cell synaptic terminals there was no response to this concentration. When $[Ca^{2+}]_i$ in the pipette solution was increased to 20 μM, there was still little change in capacitance during dialysis (Fig. 3d). However, capacitance did increase when terminals were dialysed with 52 μM $[Ca^{2+}]_i$ (Fig. 3e), which is about 50-fold higher than the free $[Ca^{2+}]_i$ required to drive secretion in equivalent experiments on chromaffin cells¹³. With 52 μM $[Ca^{2+}]_i$, the average increase in capacitance following break-in was 340 ± 48 fF ($n=8$), and the peak rate of secretion averaged 8 ± 2 fF s $^{-1}$, corresponding to 100 vesicles s $^{-1}$.

Membrane retrieval is crucial for vesicle recycling in synaptic terminals^{6,7}. In our experiments, endocytosis rapidly followed exocytosis. However, the rate of recovery depended on the strength of stimulation. Following a brief depolarization (for example, Fig. 1b), capacitance recovered rapidly, with an exponential time constant of about 2 s (Fig. 4b; right panel). After a series of depolarizing pulses that caused sustained Ca^{2+} accumulation, the recovery of capacitance to baseline was much slower (Fig. 4a), with a time constant of about 30 s (Fig. 4b; left panel). This pronounced difference could not be explained

by differences in the rate of return of $[Ca^{2+}]_i$ to baseline following strong and weak stimulation; in both cases $[Ca^{2+}]_i$ recovered along an exponential time course with similar time constants (see Fig. 4c). The two rates of endocytosis may reflect two different retrieval mechanisms, one local and rapid but with limited capacity and one slower process that comes into play only with larger amounts of secretion. Given that the rate of retrieval was influenced by the strength of stimulation, the state of the vesicle pool and the rate of replenishment might be regulated in response to stimulus history or to signals received from other neurons.

Chemical synaptic transmission in the nervous system requires that neurotransmitter be released rapidly following depolarization, and the release must be focally restricted to the target neuron. The large differences we observe in the speed and calcium-dependence of capacitance responses in synaptic terminals, compared with exocytosis in other types of cell, reflect the specialization of the terminal for such rapid and focal exocytosis. In adrenal chromaffin cells, for example, secretion elicited by activation of Ca^{2+} current proceeds at a maximal rate of about $1,000$ fF s $^{-1}$ (ref. 13), or about 400 vesicles s $^{-1}$, compared with $\sim 10,000$ vesicles s $^{-1}$ in the bipolar-cell synaptic terminal. The level of $[Ca^{2+}]_i$ necessary to drive secretion in dialysis experiments was also substantially higher (~ 50 -fold) than in comparable experiments in chromaffin cells, where 1 μM free Ca^{2+} is sufficient to activate secretion maximally¹³. Finally, there is usually little or no recovery of capacitance to baseline after secretion in chromaffin cells (see, for example, ref. 13) on the timescale in which synaptic terminals show rapid and complete recovery. All these differences reflect the different functional requirements of secretion in neuroendocrine cells compared with synaptic terminals. The speed and spatial location of exocytosis are not of primary importance in neuroendocrine cells, whereas in synaptic terminals these are crucial design considerations. Similarly, membrane retrieval and vesicle recycling, which are required

to prepare the cell for repetitive secretion, are less vital for a neuroendocrine cell than for a synaptic terminal and thus proceed at different rates in the two types of cell. □

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Localization of the site of Ca^{2+} release at the level of a single sarcomere in skeletal muscle fibres

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THE development of mechanical force in skeletal muscle fibres is brought about by rapid increases in the intracellular calcium concentration (Ca^{2+} transients) which can be detected by optical methods^{1–7}. Local stimulation experiments⁸ and ultrastructural evidence^{9,10} suggest that, at a microscopic level, these Ca^{2+} transients are generated by the release of Ca^{2+} ions from the terminal cisternae of the sarcoplasmic reticulum in response to the depolarization of the transverse tubules (t-tubules)^{11–14}. Nevertheless, to date, there is no functional information on the exact location at which Ca^{2+} release takes place. The present experiments were designed to obtain direct evidence about dynamic changes in localization and microscopic distribution of Ca^{2+} in a single sarcomere using two independent novel methodologies: confocal spot detection of Ca^{2+} transients^{15,16} and Ca^{2+} imaging with pulsed laser excitation^{17,18}.

TABLE 1 Properties of cgreen-5N and rhod-2 fluorescence signals detected at the Z- and M-lines in skeletal muscle fibres

Ca^{2+} dye	$(\Delta F/F)_Z$	$(\Delta F/F)_M$	$(t_{1/2})_Z$ (ms)	$(t_{1/2})_M$ (ms)
cgreen-5N	1.68 ± 9.42 (22)	1.39 ± 0.33 (22)	1.42 ± 0.19 (22)	3.51 ± 0.52 (20)
rhod-2	14.4 ± 9.6 (25)	8.7 ± 5.2 (8)	1.71 ± 0.31 (31)	3.55 ± 0.62 (14)

$(\Delta F/F)_Z$ and $(\Delta F/F)_M$ are the normalized peak amplitudes, and $(t_{1/2})_Z$ and $(t_{1/2})_M$ are the half-times, at the Z-line and centre of the sarcomere, respectively. The values of $(t_{1/2})_Z$ obtained with rhod-2 are significantly different from those obtained with cgreen-5N ($P < 0.0004$). In contrast, M-line transients with both dyes have almost identical $(t_{1/2})_M$ values ($P > 0.8$). Values are mean $\pm$ s.d. (number of observations).

Figure 1 shows Ca^{2+} transients recorded at different locations of a single sarcomere. Figure 1a–c shows the t-tubules lying in fine columns of increased fluorescence (black arrows) as revealed using a fluorescent potentiometric dye. Note that two columns of t-tubules define the boundaries of a sarcomere and, at the same time, serve as reference for the positioning of a fine spot of laser illumination (white arrows). The traces in Fig. 1a–c were obtained immediately before the muscle fibre was stimulated to generate action potentials; these in turn generated the fluorescence transients displayed in Fig. 1d–f. Whereas the fluorescence (Ca^{2+}) transients shown in Fig. 1d and f were recorded close to a t-tubule (see Fig. 1a and c), the transient in Fig. 1e was obtained from the middle of the sarcomere (see Fig. 1b). In frog skeletal muscle, the t-tubules lie close to the Z-line¹⁹, and the M-line of the contractile proteins lies at the centre of the sarcomere. Consequently, we will call signals detected close (within 0.5 μm) to the t-tubules ‘Z-line transients’ and those recorded at the centre of the sarcomere ‘M-line transients’. Figure 1 shows that there are significant kinetic differences between Z-line and M-line transients. Note also that the $[\text{Ca}^{2+}]$ is only temporarily elevated through the sarcomere, as both Z- and M-line transients eventually return to baseline.

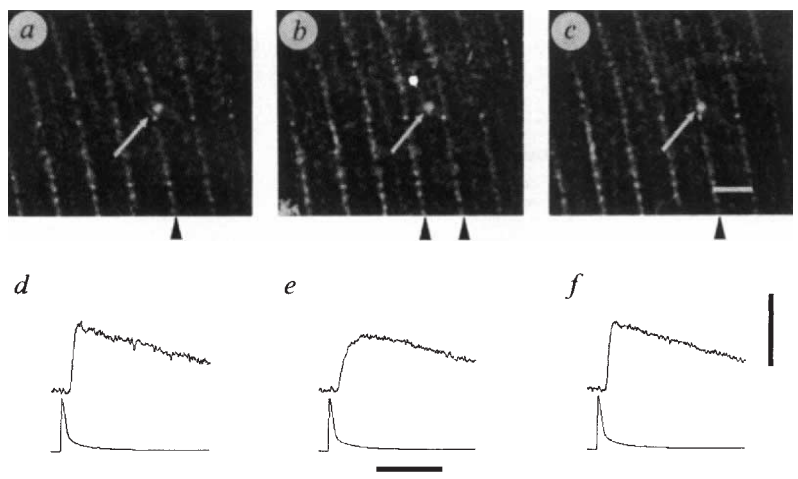
Figure 2 compares the kinetic features of Ca^{2+} transients detected at the two locations in a more expanded timescale. Superimposed Z- and M-line traces obtained in fibres stained with the Ca^{2+} dyes cgreen-5N (ref. 20) and rhod-2 (refs 12, 13, 21) are shown in Fig. 2a and b, respectively. There is an identical time delay of about 3 ms before the onset of every fluorescence transient, irrespective of the location of the recording spot and the Ca^{2+} dye used. In addition, after this constant delay, the rising phases of Z-line transients are always faster than those of M-line transients. However, Z-line transients recorded with cgreen-5N display faster-rising kinetics than those recorded at the same location with rhod-2 (Table 1). The large difference in the dissociation constants (K_{ds}) of cgreen-5N²⁰ and rhod-2^{13,21} (see Fig. 2 legend) may account for this difference. In contrast, M-line transients recorded with both dyes show similar, but slower, kinetics (Table 1). Together, these comparisons demonstrate that localized transients have significant location-dependent kinetic characteristics and few dye-dependent differences, in spite of the large differences in the dye properties.

Table 1 compares the amplitudes and kinetic properties of confocal records obtained from 11 single-fibre experiments. It is clear that, despite large differences in the amplitude of the fluorescence transients detected with each Ca^{2+} dye, the rising half-times $(t_{1/2})$ depend mostly on the location of the spot in the sarcomere. Thus, $(t_{1/2})_Z$ values recorded with both Ca^{2+} dyes are significantly shorter than $(t_{1/2})_M$ values ($P < 0.00001$). These findings strongly suggest proximity to the release site in the case of the Z-line transients and a more remote site for the M-line transients. Interestingly, however, there is no significant extra delay in the onset of the M-line transients, as would be expected

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FIG. 1 Confocal spot detection of intra-sarcomeric Ca^{2+} transients. A laser beam was focused into minute areas of a frog skeletal muscle fibre stained intracellularly with 500 μM of the Ca^{2+} dye cgreen-5N²⁰. They are displayed as fluorescence spots (white arrows) in the images shown in a–c. The position of the t-tubules was determined using the potentiometric dye RH-795 (ref. 28). They can be observed as fine columns of increased fluorescence (black arrows, a–c). The faint fluorescence in the middle of the sarcomere is caused by spectral overlap between cgreen-5N (homogeneously staining the sarcomeric volume) and RH-795. Scale bar in c, 4 μm . Changes in the epifluorescence of the Ca^{2+} dye at the spot, caused by electrical stimulation of the muscle fibre, were detected with a PIN photodiode (with a detection area of 0.008 mm^2) centred on the spot image (upper traces; d–f). Every fluorescence record is matched with its respective action potential (lower traces; d–f). Time calibration bar in e, 20 ms for traces in d–f. The vertical bar in f gives $\Delta F/F$ and membrane potential calibrations of 2 and 150 mV, respectively.

METHODS. An inverted double Vaseline gap chamber¹⁴ was used for simultaneous recording of high-magnification images, electrical activity and localized detection of fluorescence transients in single skeletal muscle fibres dissected from the semitendinosus muscle of the bullfrog *Rana catesbeiana*. The cut ends of the fibres were bathed in an internal solution containing: 110 mM K-maspartate; 20 mM K-MOPS; 5 mM sodium phosphocreatine, 5 mM $\text{Na}_2\text{-ATP}$, 150 μM EGTA, 3 mM MgCl_2 , 0.1 mg ml^{-1} creatine kinase, pH 7.0, 245 mosmols. The Ca^{2+} dyes rhod-2 and cgreen-5N were added to the internal solution at the concentrations indicated in each experiment. To prevent movement, the muscle fibres were stretched to a sarcomere spacing of 3.8–4.0 μm . They were externally perfused in the central pool with Ringer's solution at 15 °C and also stained extracellularly with 10 μM of the potentiometric dye RH-795. The chamber was palced on the stage of an inverted fluorescence microscope (Zeiss) modified for confocal spot detection, and a Nikon Fluor100 (NA 1.3) objective was used to form



the image of the muscle fibre. A xenon lamp provided whole-field epillumination for the dye RH-795 ($\lambda_{\text{excitation}}$: 570 nm; $\lambda_{\text{emission}}$: 640 nm). The raw images were obtained with a cooled CCD camera (MCD220; Spectra Source, Agoura Hills, California) and deblurred²⁹ to yield the images displayed in a–c. An argon laser (Model 80; Lexel, California) was focused to illuminate a 0.5- μm spot of the fibre. The local Ca^{2+} transients were detected by a PIN photodiode connected to a patch-clamp amplifier with 50 G Ω feedback (Axon Instruments, CV-1A, Foster City, California). The depth of field³⁰ of the spot confocal detection system was measured as 1.1 μm . The optical analog signals were acquired, simultaneously with the membrane voltage, by a data acquisition system (Axon Instruments). The fluorescence before stimulation of the muscle fibres was used to calculate the $\Delta F/F$ values. Potentiometric and Ca^{2+} dyes were obtained from Molecular Probes (Eugene, Oregon).

from the diffusional distance ($\sim 2 \mu\text{m}$) separating the two detection spots.

To investigate whether these kinetic differences result in $[\text{Ca}^{2+}]$ gradients detectable in register with sarcomeric structures, we performed experiments using a pulsed laser imaging setup¹⁸. 'Snapshot' images obtained at short times after stimulation (for example, 4 ms; Fig. 3b) show a massive increase in Ca^{2+} -depend-

ent fluorescence compared with the fluorescence of the resting muscle fibre. The image in Fig. 3b also reflects large inhomogeneities in the distribution of Ca^{2+} in the sarcomere, not seen in Fig. 3a. The high-fluorescence bands show the same periodicity as sarcomeric structures visualized with bright-field illumination (Fig. 3d). Moreover, it is clear that the banded appearance in the fluorescence snapshot images disappears 10 ms after stimulation

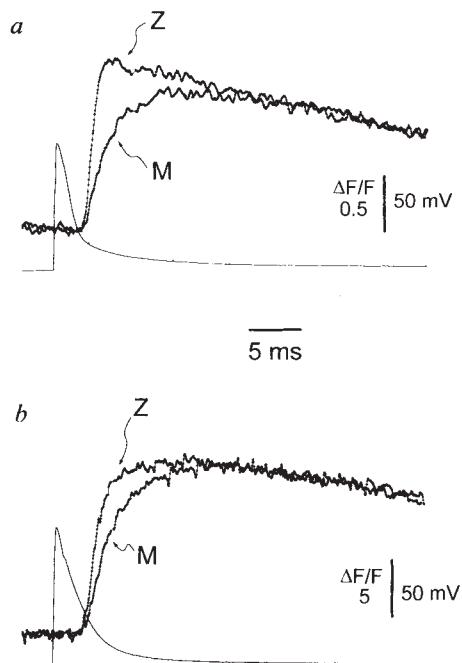
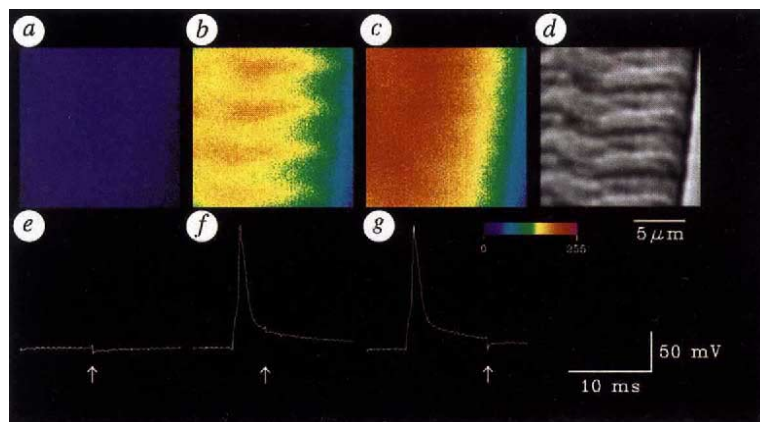


FIG. 2 a, Superposition of Z- and M-line transients (traces Z and M, respectively) elicited by action potentials (lower trace) recorded from a fibre stained with 500 μM cgreen-5N. The two single sweep traces were recorded consecutively at alternate positions of the spot, ensuring that the kinetic differences were dependent only on the spot position in the sarcomere and not on the order of sampling. b, Records obtained from a similar experiment using 300 μM rhod-2. The K_d values, obtained by fitting cuvette data to a saturation formula¹³, were 45 μM and 1.3 μM for cgreen-5N and rhod-2, respectively. The ratio between maximal to minimal fluorescence ($F_{\text{max}}/F_{\text{min}}$) was 10 for cgreen-5N and 143 for rhod-2.

FIG. 3 a–c, A sequence of pulsed laser ('snapshot') fluorescence images of a muscle fibre stained intracellularly with 300 μ M rhod-2. They were obtained by delivering a 300-ns flash to the muscle fibre at rest, and 4 ms and 10 ms after stimulation (a, b and c, respectively). The analog traces in panels e–g are records of the action potentials and show the flash delivery artefact. The colour bar shows the arbitrary pseudo-colour rendering of the fluorescence intensity for a linear scale from 0 to 256. d was obtained before electrical stimulation using bright-field illumination. The dye laser was triggered with different delays with respect to the electrical stimulation of the fibre (white arrows).

METHODS. A single muscle fibre was mounted using the procedure described in Fig. 1 legend. The recording chamber was placed instead on an optical sectioning microscope^{18,29} equipped with a high-intensity (480 mJ at 560 nm) pulsed coaxial flash lamp dye laser (Model LS-1400, Luminex; 300 ns flash duration). A light guide coupled the laser to the epifluorescence port of the microscope, where a lens focused the light so as to illuminate the entire field of view. A Nikon 100 $\times$ (NA 1.3) objective was used to view the muscle fibre.



(Fig. 3c) whereas the overall fluorescence is still largely increased. The fluorescence images in Fig. 3 give an independent 'visualization', in a two-dimensional view, of the results presented in Figs 1 and 2. Namely, snapshot detection of the fluorescence image 4 ms after stimulation indicates a fluorescence pattern in which the high intensities must be centred on the Z-lines. In contrast, the snapshot image at 10 ms suggests that throughout the sarcomere there is homogeneously high $[Ca^{2+}]$. An additional point of agreement between the Ca^{2+} distribution reported in the snapshot images and the kinetic properties of the fluorescence records is that at 4 ms, although the fluorescence is higher at the Z-line than at the centre of the sarcomere, in the latter region it is significantly higher than the basal level.

Our results provide the first direct experimental support for the hypothesis that, in skeletal muscle fibres, the primary Ca^{2+} -release sites are located at the terminal cisternae of the sarcoplasmic reticulum. The detection of out-of-focus information²² limits our ability to distinguish between kinetic events occurring in close proximity. Nevertheless, this limitation cannot readily explain why M- and Z-line transients may share a common origin (with no extra lag) and very different kinetics. Instead, our results suggest the interesting possibility that a broad band of sarcoplasmic reticulum may participate in the release process; this possibility is supported by the presence of extra-junctional Ca^{2+} -release channels in skeletal muscle²³. Finally, the present experiments describe new methodologies that are ideal for locating sites of fast Ca^{2+} release in any biological preparation, providing an alternative technique when other imaging methods^{6,24–27} fail. □

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Terminal pattern elements in *Drosophila* embryo induced by the torso-like protein

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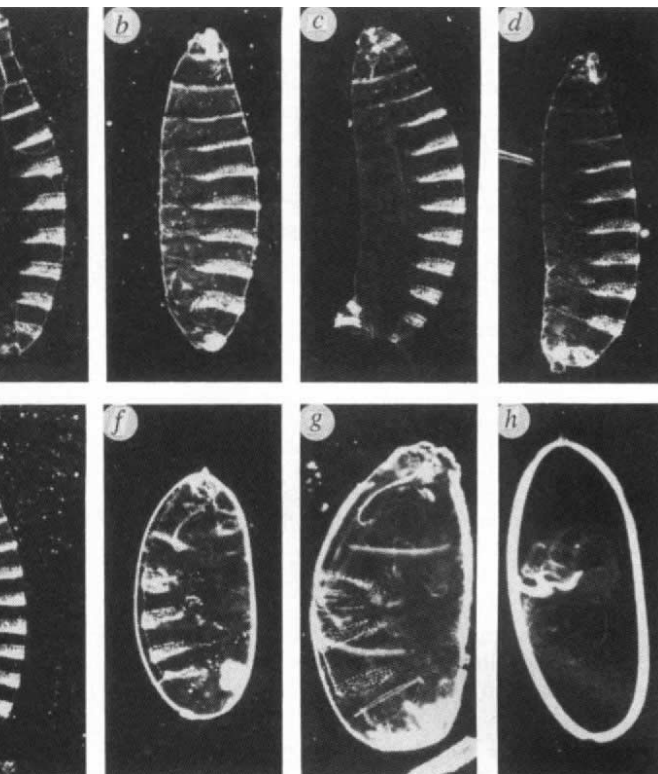
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THE genes *torso* (*tor*)¹ and *torso-like* (*tsl*)² are two of the *Drosophila* maternal group genes implicated in a receptor tyrosine kinase signalling pathway that specifies terminal cell fate (reviewed in ref. 3). Loss-of-function mutations in these loci cause an identical phenotype in which pattern elements from the anterior (acron) and posterior (telson) ends have been deleted. We have cloned the *tsl* gene and demonstrate here that, in agreement with previous genetic data, it encodes a protein that is secreted and whose transcription is restricted to specialized categories of follicle cells localized at the poles of the egg chamber. At early blastoderm stage, *tsl* protein forms a symmetrical concentration gradient at the poles on the surface of the devitelinated embryo. Unrestricted expression of the *tsl* protein in *tsl* female mutants induces terminal pattern elements and suppresses the formation of abdomen in embryos. These results suggest that the *tsl* protein is the ligand that binds to the *torso* receptor.

The *torso* gene encodes a putative transmembrane protein that is similar to receptor tyrosine kinases⁴. The *torso* protein is expressed uniformly on the surface of all early embryonic cells, but is activated only at the poles of the embryo^{5–7}. Consistent with the prediction of a spatially localized ligand in the receptor

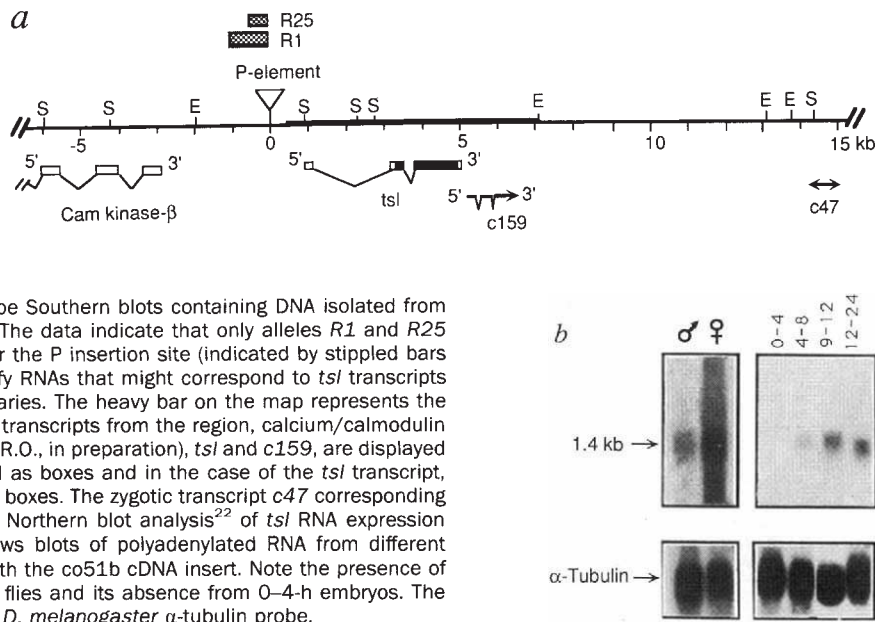
FIG. 1 a–c, Cuticle pattern in wild-type and mutant embryos. Dark-field photographs of cuticle preparations¹⁴, with embryo anterior positioned towards the top of the photograph. a, Wild-type cuticle pattern; note the internal well-differentiated head skeleton (c, acron) at the top. Each of the three thoracic and eight abdominal segments has a characteristic belt of hairs. In the tail (telson) the pair of posterior spiracles with filzkörper are visible at the terminalia (f, filzkörper; a, anal pads). b, Larvae derived from a *tsl604* mother which lack the anterior-most head structures as well as all structures posterior to abdominal segment 7 (abdominal segment 8, tuft, anal pads, spiracles and filzkörper). c, The zygotic lethal mutation *tête* (*tê*) in which the entire pattern is normal except for the head skeleton (J.-R.M. and R.O., unpublished results). d, e, Effect of *co51b* RNA injection into the posterior region of *tsl604* early embryos. d, The larvae exhibit weak posterior rescue with restoration of only an incomplete pair of non-stretched filzkörper. Note that this pattern is never observed in a *tsl* weak mutation: the presence of filzkörper and the absence of the abdominal denticle 8. e, A complete set of posterior pattern elements is restored in this larva. f–h, Phenotypes obtained from unrestricted expression of *tsl* during oogenesis. Larvae derived from (*hsp-tsl*) *tsl* females that were heat shocked at 37 °C for 1 h are shown: a series of *spliced* phenotype with increasing deletion of abdominal segments³ (f, g). h, An embryo (inverted in the egg case) with an extreme *spliced* phenotype. Similar embryos are obtained following heat-shock treatment of (*hsp-tsl*) wild-type females but not with wild-type female controls (not shown). We noted the presence of multiple filzkörper in 2% of the *spliced-like* embryos and few essentially wild-type phenotype embryos (data not shown).

METHODS. P-element and triethylenemelamine (TEM) mutagenesis was carried out according to standard procedures^{18,19}. The mutations were ordered by complementation analysis in two groups: four maternal-effect mutations *tsl604*, *tslR1*, *tslR2* and *tslR25*; eight zygotic lethals (*tê*) *R5*, *R8*, *R200*, *R214*, *TE11*, *TE44*, *TE55*, *TE99*; five zygotic lethal and maternal-effect mutations *R4*, *R7*, *R9*, *R23*, *R15*; 12 wild-type P-revertants *R30* to *R41*; *R*, P-induced revertant, *TE*, TEM-induced. Messenger RNA injection assay: RNA was synthesized *in vitro* from pBluescript *co51b* template or CaM kinase- β and injected into 90 recipient eggs derived from *tsl604* and *tslR2* at the posterior pole before pole cell formation^{6,20}. After 48 h, recipients were analysed¹⁴. We generated a construct carrying the Hsp70 promoter linked to the *tsl*-coding region by cloning the *EcoRI* *co51b* insert in the pCaSpeR-hs (ref. 21). pCaSpeR-hs β tsl was introduced by P-element-mediated transformation into *w¹¹¹⁸* flies. Six independent lines (*hsp-tsl*) were obtained;



(*hsp-tsl*)*tsl604* and (*hsp-tsl*)*tslR2* females were generated by standard genetic techniques. Females of the various genotypes tested were treated by a 1-h 37 °C heat shock. Embryos laid after this treatment were collected every 2 h from 0 to 60 h. We found about 50% of non-fertilized eggs but 100% of fully differentiated larvae display filzkörper, and about 90% a *spliced* phenotype from the collections of 6 to at least 60 h after heat shock. However, few almost wild-type larvae (wild type apart from the weak rescue of the head defects) are observed at 6–10 h and 40 h after heat shock. In control experiments made with (*Hsp*-CaM kinase- β) *tsl604* and *tslR2* lines treated by a 1-h heat shock, no larvae showed the abdominal denticle belt 8, anal pads or filzkörper. Only 10% of the embryos failed to develop or showed unrelated defects superimposed on the *tsl* phenotype.

FIG. 2 a, Genetic and physical map of the *tsl* region. A restriction map of 20 kb of the *tsl* region is illustrated with the following restriction sites: E, *EcoRI*; S, *Sall*. The zero is defined by the P-element insertion site represented by a triangle. To isolate DNA flanking the P insertion at 93E, an EMBL3 library of DNA isolated from a homozygous *tsl604* strain was screened for clones containing the P-element. A chromosome walk of about 150 kb around the site of the P-white insertion was made. Subclones containing 14-kb fragments from the region defined by the *tsl604* P insertion were used to probe Southern blots containing DNA isolated from the three strict maternal *tsl* alleles (Fig. 1 legend). The data indicate that only alleles *R1* and *R25* have deletions, of 1.5 and 0.7 kb, respectively, near the P insertion site (indicated by stippled bars at the top). The subclones were also used to identify RNAs that might correspond to *tsl* transcripts on northern blots of poly(A)⁺ RNA from wild-type ovaries. The heavy bar on the map represents the region sequenced in this report. The three maternal transcripts from the region, calcium/calmodulin protein kinase- β subunit (CaM kinase- β) (J.-R.M. and R.O., in preparation), *tsl* and *c159*, are displayed below the physical map with the exons represented as boxes and in the case of the *tsl* transcript, the coding regions of the exons are denoted by solid boxes. The zygotic transcript *c47* corresponding to the *tête* gene is only approximately localized. b, Northern blot analysis²² of *tsl* RNA expression during embryo development. The upper panel shows blots of polyadenylated RNA from different developmental stages of the embryos, hybridized with the *co51b* cDNA insert. Note the presence of the 1.45-kb mRNA in 4–12-h embryos and in adult flies and its absence from 0–4-h embryos. The lower panel shows the same filter hybridized with a *D. melanogaster* α -tubulin probe.



a

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M 1

GCTGAGCAGTCGCTGGCTGGCTGGCTTTTCTGGCTGTGACCTGGCTGGCTTCTGGCGGATGGAGGTGCTGCGAGCTCCACGCTCCGAGTCGGCAGCGCC
L S D A S W P G L F W L T L L A L L D G * G R R S Q L R I G K A 34

2422 ATCAATATATCTCTGGCTATGGCTACCTGGGAATCTCCATCGGTGTGATACCGCTCAATGACAACCTCGGAGCCGATCGCTGGGTGTTTAAAGGACCCA
I N I F L R Y G Y L G I S M R V I P L N D N S G P D R W F V K E P T 68

CCAAAACACTCTATAGGCTaagggactaagggcgatgaagcttcaatttaaaataaantggcgctgatgaactcggaatttgttgtataaccttt

K N I Y R 73

2622 aaccacaataccataattatttggagataatacaaaattttaaaatagggtttaaagttgttataccttaaaactatttttagttgtaatttatattgtt
cacttcgcgcacggtttgtcgtagaattgagcgcttattattttgtttgatcagatttaggaataacgactcattatacgtatttcaagttatgctcactt
2822 gatattttactcgtataatttagctgactgactagacaattagataaattatgatttttttcttcagAATCTGAGCTGGCGGAGCATAGGACAC

N L S G L A E S H E D 85

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T P G I F H G D F H M E F C E N R R Q L F Q A Y F R D F S I E R M 118

3022 GACAAACCGTGGGAGCAATTTACTGCGGATGTTTTCGCGAATATCGGCCAAGAAGCTGGGATCAATACATCTTTTCACTCAAGGCGATCTACTGCTATG
D K P W E A F T G G W F P D N A A K K L G I N T S F I Q G D Y S Y V 152

TTTTGCTGAGGCTGGCTGGCTTCAGGGAACGGGGCGTCTTAACGCTGAGATTCCGCTGCACCAACCTTGGAGCCGAGTGTGCGATCGAGAATGGATCA
L V R V R V R A F T G R L N A E I P V H Q P L E P D V R S R M D Q 185

3222 ACTGCAAAATCGAAATATACCTTCGACGTCGCAATTTATGGAAGATGTGGGCACCACTACGTAAATCTATACACCACCGCAATTCGCTGTATCAGGTG
L Q I G I N T S A V R F M E D V G T H V Y N S Y T T G N S L Y Q V 218

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F V Y S R K N Y S M I K E R I K S K G L N G L S K L D L Y N F A P 252

3422 CCTGTGTCGCCCATCTGGGCCAGATTCGATCGSCTAGTGCCAATGCTACAGTGGAAAGATGGGCGAGAAGCAAGCTGCAGTACGAGTACTATTGGT
W F A A H L G Q I R S A S A N A T V E R W A R R K L Q Y E Y V V 285

CAAGTATGTGACCTCTGAAACTCGAGCTGATATAGTACTTACTGCGATCCCTGGACTCGCTGCGGCAAGATGCCATCTGCAGTGTGGACCTCAAG
K Y V T L L K L H G N S T L L R S L S G L L G N D A I L Q L D L K 318

3622 TCGCTGAAGCCATCTTCGGGAGGAGCGGGAAGGAGAGGCTGTGACCACAGGTTTTCGCAACAACATGTGAAGCTCTGGGAGCTTAACATGGCCGAGA
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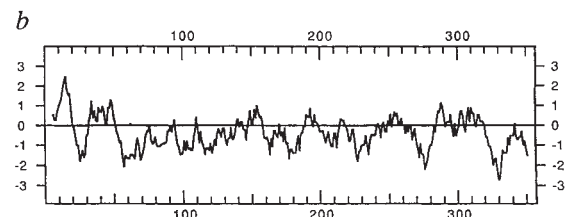
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H P T R - 356

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4222 cgttggtgatttttcaaacggtttcatcagctcagctgaactgaatccgaataagcagtcggtttgtattttgattttgatttgggttgggtataatactggc
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FIG. 3 a, Genomic and cDNA nucleotide sequence of the *tsl* gene and the deduced sequence of the *torso-like* protein. The exon sequences are listed in capital letters and the intron and flanking sequences in lower-case letters. The deduced amino-acid sequence is in one-letter code. The putative signal peptide cleavage site, predicted by the method of von Heijne¹³, is indicated by an asterisk and possible N-linked glycosylation sites are underlined. The boxed amino acids correspond to leucine zipper. The boxed nucleotide sequence indicates the non-canonical poly(A) addition site which is identical to that of the D-int-1 secreted protein²³. b, Hydropathicity profile of the deduced amino-acid sequence of the 356-amino-acid *tsl* protein sequence determined by the method of Kyte and Doolittle²⁴.

METHODS. 6.5 Kilobases of genomic sequence and the 1.45-kb co51b cDNA were sequenced completely on both strands. DNA fragments were subcloned into pBluescript and nested deletions were generated by exonuclease III digestion. Dideoxy sequencing using Sequenase (USB) was performed according to the manufacturer's instructions. Oligonucleotides corresponding to previously determined sequences were used in some sequencing reactions. Sequence analysis was carried out using DNA Strider software.

tyrosine kinase model, mosaic analysis experiments demonstrated that *tsl* expression is required only in a category of specialized follicle cells located at the egg shell poles⁸. To test this model, we selected the *torso-like* allele *tsl604*, induced by P-white in a screen for female sterile mutations. This mutation, which is allelic to *tsl035* (ref. 2), displays a strict maternal phenotype in which the anterior head skeleton is truncated and the structures posterior to the abdominal segment 7 do not form (Fig. 1a, b). The DNA flanking the P-white insertion was isolated (Fig. 2a) and DNA probes from the *tsl* region identified three maternal



transcripts. Probes for each of the transcripts were used to examine the expression pattern of the RNA by digoxigenin *in situ* hybridization of whole-mount egg chambers⁹. An *EcoRI-SalI* fragment which recognizes a minor, 1.45-kilobase (kb) maternal messenger RNA specifically labels the border and the polar follicle cells in a pattern predicted for *tsl* by previous genetic studies^{8,10}. This fragment was selected for further study and the two other maternal transcripts, those for calcium/calmodulin protein kinase type- β (CaM kinase- β) and *c159*, were eliminated on the basis of their expression in nurse cells (data not shown).

DNAs complementary¹¹ to the ovary mRNA detected by the *EcoRI-SalI* fragment of the *tsl* locus were isolated. The sequence corresponding to 1,450 base pairs (bp) of the *tsl* transcript which spans about 3.5 kb of genomic DNA was determined from the cDNA co51b. Fragments of cDNA co51b hybridize to a unique 1.45-kb transcript in poly(A)⁺ RNAs from females, males, ovaries and 4–12-h embryos (Fig. 2b).

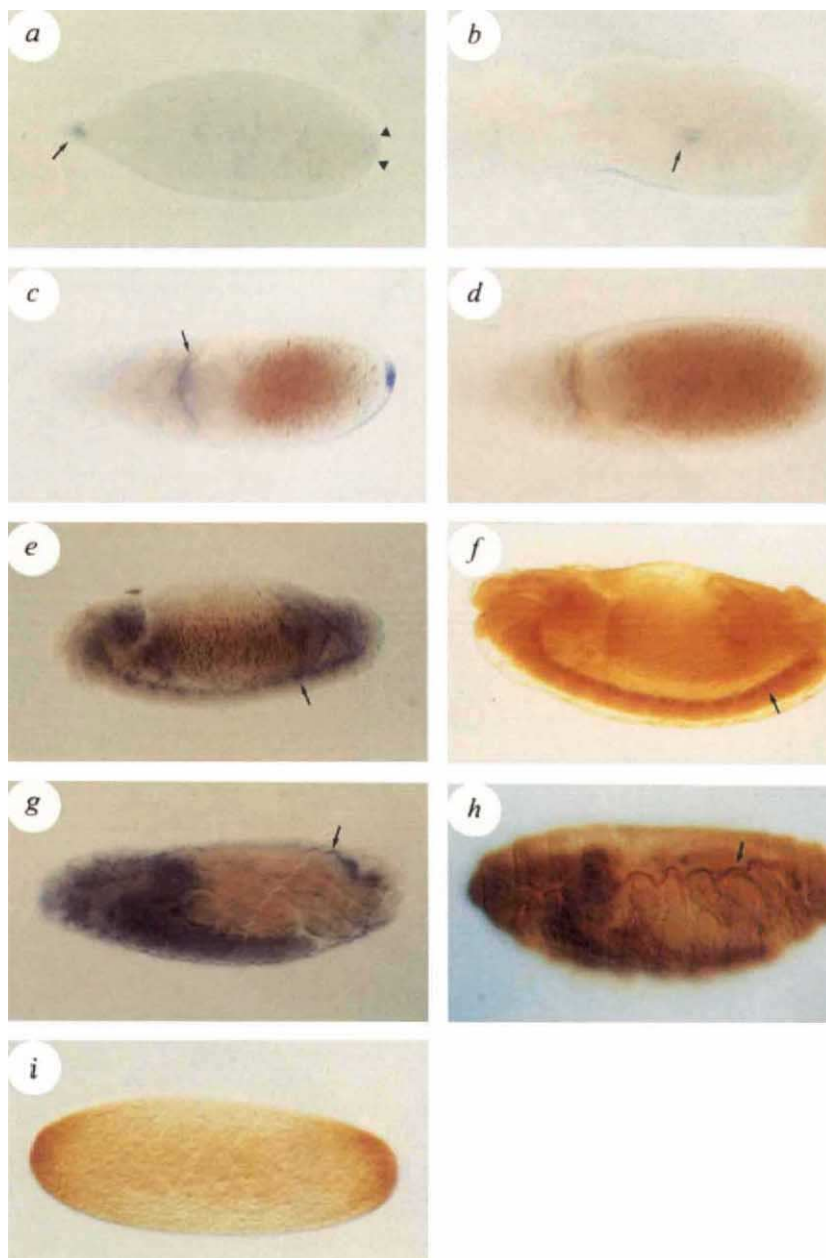
The sequence of cDNA co51b contains a 356-codon open reading frame (ORF) capable of encoding a polypeptide of relative molecular mass (M_r) 41,593 (Fig. 3a). The ORF spans part of the second and third exons. This ORF is preceded by a stop codon and is initiated by the second methionine codon downstream from the predicted transcription initiation site (position

125). The sequence surrounding this AUG codon only weakly matches the *Drosophila* consensus translation initiation site¹². The deduced protein is basic, containing a remarkable 11% leucine and 12% lysine plus arginine residues, giving a pI of 10.07. The sequence also contains a unique cysteine that could serve for dimerization and a single leucine zipper motif. A hydropathy profile of the predicted *tsl* polypeptide (Fig. 3b) reveals a unique, strongly hydrophobic region located at the amino terminus of the protein. This region conforms to the rules defining a signal sequence and, based on the criteria of Von Heijne¹³, there is a potential signal peptidase cleavage site.

To confirm that the 1.45-kb mRNA is the *tsl* transcript, RNA transcripts synthesized *in vitro* from *tsl* complementary DNA were tested for their ability to provide *tsl* function when injected into embryos from *tsl604*-containing females. The *tsl*-containing eggs were injected with the synthetic RNA at 0–10% egg length and the developed embryos analysed¹⁴. The specific *torso-like* tail defect in *tsl604* embryos can be completely corrected by injection, before pole cell formation, of co51b RNA, but not CaM kinase- β RNA, into the eggs. More than 50% of the differentiated embryos made the telson elements filzkörper, and 20% also made anal pads, tufts and the seventh and eighth ventral denticle belts (Fig. 1d, e). This functional complementation of

FIG. 4 a–d, Pattern of *tsl* mRNA distribution during oogenesis. In all panels the *tsl* mRNA was detected by *in situ* hybridization using labelled co51b cDNA as probe and an alkaline phosphatase detection system⁹. In all figures, anterior is to the left. a, Stage 8, blue staining is concentrated at the anterior pole in border cells that have not yet started to migrate; the label is also seen in the polar and flanking follicle cells at the posterior pole. b, Stage 9, staining is in the migrating border cells and in the posterior pole (which is out of focus in this photograph). c, Stage 10, border cells have completed their migration. Focus is made to show staining in the centripetally migrating border cells. d, A stage-12 egg chamber. The nurse cells are degenerating; bipolar staining is still seen. e–i, Spatial localization of *tsl* RNA and protein in wild-type embryos. RNA distribution is shown in e and g and protein distribution is shown in f and h. Anterior is to the left and ventral is down. The pattern of *torso-like* protein and RNA distribution coincide, except for a slight delay in protein accumulation. e–h, Lateral view of embryo after germ-band shortening¹⁵. The segmented nervous system and the brain are labelled. g, h, Strong expression in the cells forming the tracheal system. i, Spatial distribution of the *torso-like* protein in early embryo. *tsl* protein is observed at the surface of the syncytial blastoderm embryo (stage 14) in the form of symmetrical caps. The staining forms a concentration gradient.

METHODS. To raise antibodies against the *tsl* protein, the cDNA co51b was inserted into the pATH2 translation vector. The fusion protein was used to raise rabbit polyclonal antibodies. Staining of whole-mount embryos with affinity-purified *tsl* protein antibodies was performed by standard methods²⁵.



the *tsl* phenotype proves that the cloned cDNA encodes the biological activity required for terminal pattern formation. In addition, it is interesting that the *torso-like* protein does not need to be localized in the polar follicle cells to function. One likely explanation is that *tsl* passes beyond the egg membrane in both directions.

To examine the localization of *tsl* RNA in ovaries and embryos, whole mounts were hybridized *in situ*^{10,15}. The *tsl* RNA first appears in the vitellarium at about stage 8, when bipolar staining is observed at the anterior pole of the egg chamber, in the border cells just before they begin to migrate, and at the posterior pole in the polar and flanking follicle cells (Fig. 4). Throughout oogenesis, the accumulation of *tsl* RNA is restricted to follicle cells. Stage 9 staining persists in migrating border cells and at the posterior pole in follicle cells. At stage 10, border cells have completed their migration and continue to express *tsl* together with polar posterior follicle cells. *tsl* RNA is also seen in centripetal follicle cells. This profile of expression is conserved until the egg is laid. We were surprised to discover that *tsl* is highly expressed in the central nervous and tracheal systems during embryogenesis¹⁵. The Tsl protein is detected at the beginning of nerve cell segregation, but only late in the formation of the tracheal system. As none of the *tsl* alleles analysed here was a null mutation, additional experiments are needed to determine whether or not *tsl* is essential during neurogenesis or tracheal system development.

To define the cellular location of the *tsl* protein, polyclonal rabbit serum against a bacterially expressed fusion protein derived from *tsl* cDNA co51b was produced and affinity purified. This antiserum specifically recognizes the *torso-like* protein based on the similarity between the pattern of mRNA and protein expression in embryos (Fig. 4). *tsl* protein expression peaks at the early syncytial blastoderm (stage 4) and nuclear cycle 12 (Fig. 4i). The *tsl* protein is localized at the embryo poles, extending roughly over 0–17% egg length in the posterior and 83–100% egg length at the anterior. This staining forms a symmetrical mirror-image gradient on the surface of the devitellinized embryo. The anterior and posterior caps are observed even when mechanical devitellinization is performed before paraformaldehyde fixation. Thus the *tsl* protein is secreted from the polar follicular cells into the periplasmic space where, after a sequence of unknown events, it diffuses and then becomes associated with the surface of the embryo.

Another crucial question is whether the other components necessary for the activation of *torso* are also localized with *tsl* or are distributed uniformly in the embryo. To investigate this question, a P-element construct, in which *tsl* is under the control of the heat-inducible Hsp70 promoter, was made and used to establish six independent transgenic lines of flies. The consequences of ectopic expression of the *tsl* protein during oogenesis were observed on cuticular differentiation in embryos derived from homozygous *tsl* mothers. These (*hsp-tsl*) *tsl* embryos do not make anal pads or filzkörper. However, when the adult females were heat shocked, ubiquitous *torso-like* expression was seen in early embryos by antibody staining (data not shown). The developing embryos produce anal pads and filzkörper but delete the contiguous abdominal segments, giving a phenotype similar to that observed with the *torso* gain-of-function allele *spliced* (Fig. 1f–h). The *spliced* phenotype is also obtained when *hsp-tsl* expression is activated in a wild type, rather than the *tsl* background. These results indicate that overexpression of *tsl* can activate *torso* in all regions of the embryo, implying that all the components necessary for activation are uniformly present in the periplasmic fluid.

Genetic studies⁸ demonstrated that, in contrast to the three upstream *torso* genes *trunk* (ref. 1), *fs(1) Nasrat* (ref. 16) and *fs(1) pole hole*¹⁷, which are germ-line dependent, *tsl* is follicle dependent. The *in situ* hybridization experiments reported here demonstrate that the *tsl* transcript is, in fact, highly localized and that the *tsl* protein forms an apparent symmetrical concentration

gradient at the termini. The observed concentration gradient of *tsl* protein molecules (Fig. 4i) might create a postulated gradient of activated *torso* protein molecules⁵. If localization is due to limited binding of the diffusible ligand to a large excess of *torso*, the corollary of this property implies that among the upstream genes, indiscriminate expression of the gene encoding the ligand should lead to ubiquitous activation of the *torso* protein and result in a *spliced* phenotype. Consistent with this prediction, we were able to create *spliced* embryos by inducing uniform distribution of saturating quantities of the *tsl* protein in *hsp-tsl* female transgenic flies. The properties of the *tsl* gene product presented here are consistent with the notion that the *tsl* protein is the ligand for the *torso* receptor. Nonetheless, definitive proof of this must await physical binding experiments. □

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The nucleus is insulated from large cytosolic calcium ion changes

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EXTRACELLULAR events regulate functions in the cell nucleus by means of calcium ions acting through effector enzymes^{1–5}. Recently, the traditional view of the nuclear pore as freely permeable to small ions^{6,7} has been questioned as a result of reports that nuclear calcium can be regulated independently of cytosolic calcium^{8–12}. We have used confocal microscopy of fluorescent Ca²⁺ indicators to investigate the Ca²⁺ dynamics between cytosol and nucleus in neurons. We find that a previously reported amplifica-

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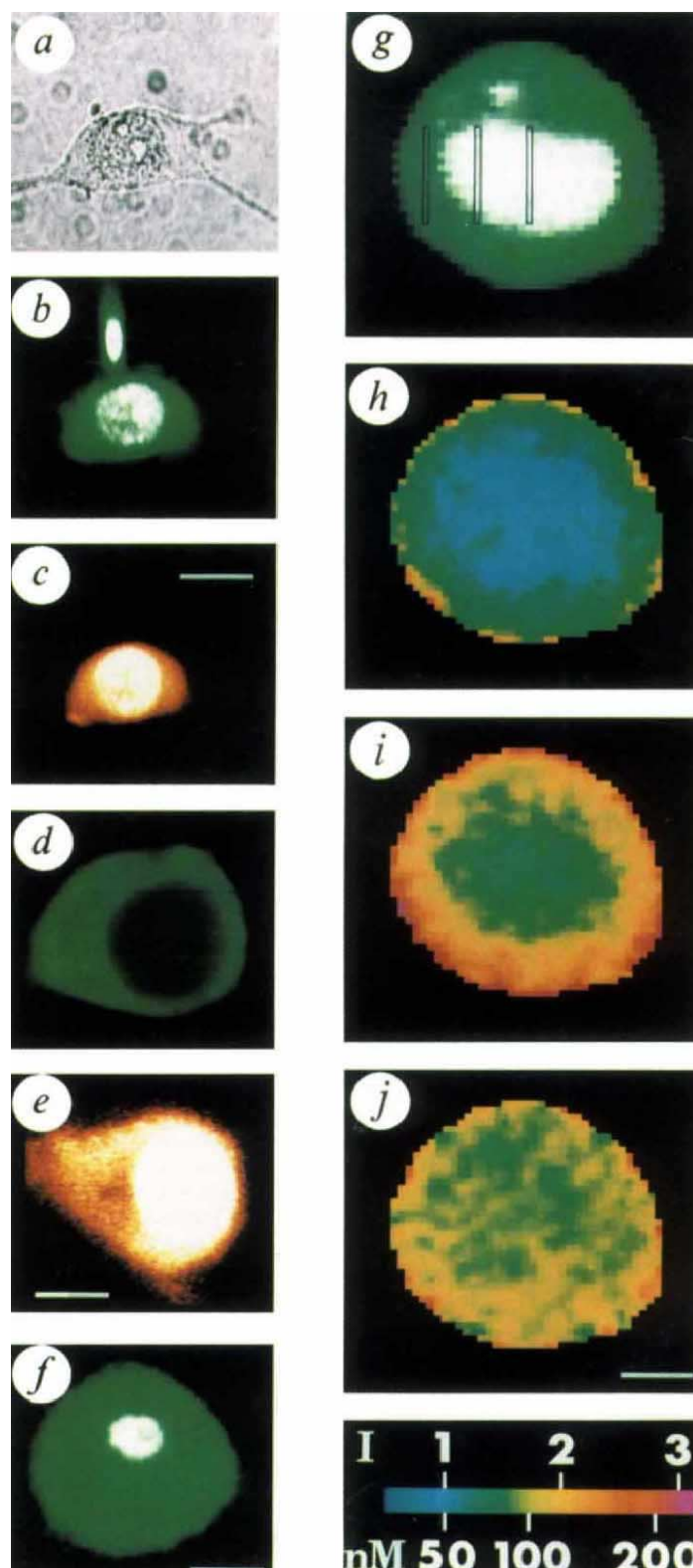
tion of Ca^{2+} changes in the nucleus^{13–16} is a measurement artefact. Small changes of cytosolic Ca^{2+} cause equally rapid changes in nuclear Ca^{2+} , consistent with the free diffusion of Ca^{2+} through nuclear pores. In contrast, large cytosolic Ca^{2+} increases (above 300 nM) are attenuated in the nucleus. Our results show the nuclear envelope shapes but does not block the passage of Ca^{2+} signals from cytosol to nucleus.

The nucleus of mouse neuroblastoma cells was visible in phase contrast microscopy (Fig. 1a) and stained brightly with ethidium bromide (Fig. 1c). The Ca^{2+} indicator dye Fluo-3 (ref. 17), intro-

duced into the cell by a patch pipette, gave the strongest signal from the nucleus (white region in Fig. 1b). A lower signal from cytosol than from nucleus is a common feature of cells loaded with hydrophilic dye^{6,13} and is due in part to exclusion of dye from membrane-bound organelles that are absent in the nucleus¹⁸. Depolarization for 1 s under voltage clamp caused a Ca^{2+} influx through voltage-gated channels. To display the resulting fluorescence changes, we divided the fluorescence at each point by the fluorescence before depolarization and displayed the result using a colour scale (Fig. 1h–j). Calcium influx

FIG. 1 Small changes of Ca^{2+} easily pass the nuclear envelope. a–c, The same cell viewed by phase contrast (a), Fluo-3 fluorescence (excitation 488 nm, emission ≥ 530 nm) (b) and fluorescence of the nuclear dye ethidium bromide (excitation 514 nm, emission ≥ 590 nm) (c). We display fluorescence images using a palette that displays intermediate intensities in colour (orange for ethidium, green for fluorescein analogues) while regions of brightest fluorescence appear white. The bright object above the cell in b is a confocal section of the patch pipette. Scale bar in c, 20 μm . d, The nuclear envelope of a patch-clamped cell excludes fluorescein–BSA (1 mg ml^{-1}) included with the pipette solution. Image acquired 1 h after patch rupture. e, The nuclear envelope is permeable to the ethidium homodimer 1 ion (included in the pipette as chloride, 100 μM). Scale bar (10 μm) in e applies to d, e. f, The nuclear envelope is permeable to injected dextran-conjugated Calcium Green (M_r 10,000). Scale bar, 20 μm . g, Fluo-3 fluorescence in a fifth cell, the brighter nucleus appears white. h–j, Normalized fluorescence (I) in the same cell during (h, i) and after (j) voltage-clamp depolarization displayed using the colour scale at the bottom. Scale bar in j, 10 μm . Calcium values below the bar are calculated as in Fig. 3 legend (137, 1,053 and 1,970 ms after the onset of a 1-s depolarization from -40 mV to $+10$ mV).

METHODS. Neuroblastoma cells (a–e, g–j) and sensory neurons (f) were grown as described previously^{24,25}, except that serum was at 10%; all experiments were at 23 °C. Cells were filled with Fluo-3 via a patch pipette (Fig. 3 legend) containing 5 mM Fluo-3; later experiments used 100 μM Fluo-3. There was no difference between the results obtained using different dye concentrations, but the higher concentration allowed measurements to be taken sooner after patch rupture. Fluo-3 produced no signal with ethidium bromide filters; subsequent addition of ethidium bromide revealed that the nucleus co-localized with the Fluo-3 bright object ($n=8$). Images were acquired every 183 ms using a Leica CLSM-fluoview confocal microscope at a vertical resolution of 3 μm (FWHM, reflectance mode) and a horizontal resolution of 1 pixel per μm . Images were then spatially filtered using a 3×3 matrix (Leica 'longpass'), giving a final horizontal resolution of better than 3 μm .



generated a Ca^{2+} wave that moved radially inward, passing apparently unhindered through the nuclear envelope to raise nuclear levels to 101 ± 7 nM ($n=10$). This result fits with the traditional view of the nuclear pore as permeable to solutes of relative molecular mass $M_r < 50,000$ (ref. 6), but conflicts with recent suggestions that the nuclear envelope is a barrier to Ca^{2+} diffusion^{8,12}.

Two simple explanations might resolve this conflict. First, patch clamping might cause lysis of the nuclear envelope. However, bovine serum albumin (BSA; M_r 64,000) included in the patch pipette did not enter the nucleus (Fig. 1d) ($n=3$), excluding this possibility. Second, the nuclear pore complex might contain fixed positive charges that normally prevent passage of cations such as Ca^{2+} , but in our experiments exogenous Fluo-3 might allow Ca^{2+} to pass as the complex $[\text{Ca-Fluo-3}]^{3-}$. However, the ethidium homodimer⁴⁺ ion entered the nucleus within seconds (Fig. 1e) ($n=8$). Because this ion bears twice the positive charge of Ca^{2+} , it is unlikely that fixed charges in the pore block Ca^{2+} movement.

The complex that straddles the nuclear pore resembles a wheel, with hub, spokes and rim¹⁹. Macromolecules can only pass through the central hole, which has an effective size 9 nm across by 15 nm long⁶, but small ions would be expected to pass easily between the spokes⁷. In agreement with this prediction, the radial Ca^{2+} wave was well fitted by a simple mathematical diffusion model in which the entire area of each nuclear pore was available for Ca^{2+} diffusion. A model in which Ca^{2+} movement was restricted to the central 9-nm hole fitted the data less well (Fig. 2).

Voltage-clamp depolarizations of primary adult rat sensory neurons that raised Ca^{2+} to 300 nM or less elicited a similar pattern of Ca^{2+} diffusion, with no significant delay at the nuclear envelope. However, the amplitude of the Ca^{2+} current in sensory neurons is larger than in neuroblastoma cells, so that depolarizations lasting 1 s raised Ca^{2+} to micromolar levels. This produced a more complex pattern of Ca^{2+} diffusion (Fig. 3a–d). The radial Ca^{2+} wave was delayed at the nuclear envelope, so that nuclear Ca^{2+} remained lower than cytosolic Ca^{2+} for seconds (Fig. 3c) before equilibration. On average, nuclear and cytosolic Ca^{2+} took 3.2 ± 0.5 s ($n=9$) to reach to within 10% equilibration at which time levels were between 137 and 1,272 nM (mean

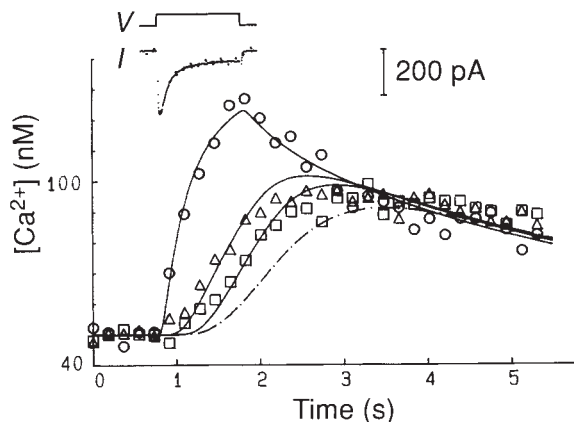
325 ± 114). When Ca^{2+} influx ceased, nuclear Ca^{2+} levels fell more slowly than cytosolic levels (time constants of decay in nucleus and cytosol were, respectively, 20.73 ± 5.045 s and 10.89 ± 1.47 s, $n=42$, different at 2% by paired *t*-test) and in a number of cells (9 of 30) remained higher than cytosolic levels for several seconds (Fig. 3e, f).

The apparent deviation of nuclear from cytosolic Ca^{2+} might result from a different behaviour of the dye in the very different chemical environment of the nucleus. To address this possibility, we calibrated Fluo-3 in the cytosol and nucleoplasm. The slight difference found (Fig. 3g) did not explain the observed attenuation of nuclear Ca^{2+} change and, when applied to data from all cells depolarized for 1 s, showed a clear attenuation of the nuclear change at concentrations above 300 nM (Fig. 4a). The simplest explanation of these data is that a high-capacity Ca^{2+} buffer in the nucleoplasm takes up Ca^{2+} at concentrations above 300 nM, to release it again as levels fall. Alternatively, it is possible that the nuclear pores partially close at Ca^{2+} concentrations above 300 nM.

Previous work using various cell types has produced a very different picture. In these studies the nucleus shows a larger Ca^{2+} change than the surrounding cytosol, implying the operation of a Ca^{2+} amplification system^{13–16}. We were concerned that patch clamping might be causing the loss of a soluble intracellular component necessary for amplification, and therefore tried an alternative method for introducing dye and depolarizing sensory neurons. We injected dextran-conjugated Calcium Green from an intracellular micropipette and immediately removed the pipette. After 30 min the 10,000- M_r dextran had entered the nucleus⁶ (Fig. 1f). When cells were depolarized with potassium we never saw nuclear Ca^{2+} amplification. Rather, small cytosolic Ca^{2+} changes were transmitted rapidly to the nucleus (Fig. 4b) and larger changes were attenuated (Fig. 4c, d). In contrast, depolarization of cells 30 min after injection of Fluo-3 (M_r 765) always elicited an apparent nuclear Ca^{2+} amplification (Fig. 4e). Small anionic dyes, but not dextran conjugates, can be sequestered in organelles²⁰. We hypothesize that Fluo-3 sequestration leads to underestimation of cytosolic Ca^{2+} changes by creating a pool of cytoplasmic dye that is insensitive to cytosolic Ca^{2+} . In the nucleus, which lacks membrane-bound organelles, Ca^{2+} is measured correctly. Three results confirmed this hypothesis.

FIG. 2 A simple model of radial diffusion fits the measured Ca^{2+} changes. *V*, Transmembrane voltage; *I*, transmembrane current. $\circ$, Δ , $\square$, Calcium calculated from fluorescence in the three areas indicated by rectangles in Fig. 1g. In 10 cells, the peak Ca^{2+} at these three regions was 166 ± 25 , 108 ± 10 and 101 ± 7 nM, while peak Ca^{2+} immediately under the membrane (calculated from fluorescence in a shell 5% as thick as the cell radius) was 205 ± 31 nM. Continuous lines are the results of a diffusion model in which the whole area of the nuclear pore is assumed available for diffusion. The dashed line corresponds to $\square$ but was calculated assuming Ca^{2+} diffusion is restricted to the central 9 nm across by 15 nm long⁶.

METHODS. Electron micrographs of 60-nm sections of neuroblastoma cells and sensory neurons, respectively, showed pore diameter as 70.8 ± 2.6 nm ($n=17$) and 60.8 ± 2.5 nm ($n=12$); nuclear envelope was 21.2 ± 0.8 nm ($n=11$) and 22.7 ± 0.8 nm ($n=18$) across. We counted 123 and 52 pores, respectively, in which lips at either side were visible (implying at least half the pore lay within the section), giving 24.0 and 13.8 pores μm^{-2} . Diffusion between 20 concentric volume elements was calculated as rate = $\Delta[\text{Ca}^{2+}] \times D \times \text{area}/\text{thickness}$, except for elements separated by the nuclear envelope where the area for diffusion was reduced to *f*% for a distance *d* of the total. Solid lines: *f*=9.45%, *d*=21.2 nm. Dashed line: *f*=0.153%, *d*=15 nm. Four parameters: *D* (3.5×10^{-11} m² s⁻¹), rates of pumping across plasma-membrane (3×10^{-7} m s⁻¹) and into organelles (0.17 s⁻¹) (both assumed proportional to Ca^{2+} excess over 50 nM) and fraction of whole-cell Ca^{2+} current appearing as free Ca^{2+} (1 in 714), were adjusted for best fit. The last term should not be taken as a measure of buffering because not all Ca^{2+} influx is into the cell body.



First, permeabilization of the plasmalemma revealed a time-dependent sequestration of Fluo-3, so that 30 min after injection more than half the fluorescence was non-cytosolic (Fig. 4f). Second, the anion pump blocker sulfinpyrazone²⁰ prevents Fluo-3 sequestration (Fig. 4f) and eliminates apparent nuclear Ca^{2+} amplification (Fig. 4g) (9 cells). Third, depolarization of sensory neurons 2–5 min after Fluo-3 injection, when sequestered dye is minimal, never gave apparent nuclear amplification (Fig. 4h) (five cells). Connor¹⁸ has shown that significant accumulation of

dye in organelles can occur during acetoxymethyl ester loading, leading to various artefacts, including apparent nuclear Ca^{2+} amplification. Our results show that the same artefact can be produced by a distinct mechanism, active dye sequestration.

Our results show that the nuclear envelope is not a barrier to Ca^{2+} . Small increases in cytosolic Ca^{2+} are transmitted rapidly to the nucleus, however the nucleus is insulated against cytosolic Ca^{2+} transients greater than about 300 nM. Thus, enzymes transients with relatively high Ca^{2+} requirements will be differ-

FIG. 3 Large cytosolic Ca^{2+} changes are attenuated in the nucleus. a, Fluo-3 fluorescence, the brighter nucleus appears white. b–d, Normalized fluorescence (*I*) in the same cell after voltage-clamp depolarization displayed using the colour scale at the bottom (137, 1,053 and 2,520 ms after onset of 1-s depolarization from -40 to +10 mV). Calcium values below the bar are calculated from the separate cytosol (C) and nuclear (N) calibrations shown in g, assuming a uniform resting Ca^{2+} of 50 nM²⁵. e, f, Example of a cell in which nuclear Ca^{2+} remained higher than cytosolic for several seconds after depolarization. e, Fluo-3 fluorescence. The bright object to the left of the nucleus is the patch pipette. f, Normalized fluorescence 6 s after onset of depolarization (1 s from -40 to -30 mV, which raised Ca^{2+} to levels that temporarily saturated the dye). Nuclear Ca^{2+} is falling more slowly and now exceeds cytosolic Ca^{2+} . The pipette, where fluorescence does not change, appears blue, normalized *I*=1. Scale bars, 20 μm . g, Fluo-3 fluorescence measured on the confocal microscope at different calcium concentrations, normalized to fluorescence in 39 μM calcium. 'Extracellular' (·····): 100 μM Fluo-3 in Ca-EGTA buffers containing 1 mM free Mg^{2+} (Molecular Probes). Dissociation constant $K_d = 377 \pm 29$, $I_{\text{sat}}/I_{\text{free}}$ (intensity with $\text{Ca}^{2+} = 39 \mu\text{M}$ /intensity with $\text{Ca}^{2+} = 0$) = 90 ± 6 (3 determinations). These parameters were applied to neuroblastoma cell measurements. 'Cytosolic' (---): 100 μM Fluo-3 in Ca-EGTA buffers was applied to sensory neurons permeabilized with 100 $\mu\text{g ml}^{-1}$ lysophosphatidylcholine. Measured parameters ($K_d = 650 \pm 59$ nM, $I_{\text{sat}}/I_{\text{free}} = 14 \pm 2$; 4 cells) were applied to sensory neuron cytosol measurements. 'Nuclear' (—): Dye in 100 μM Fluo-3 in Ca-EGTA buffers was applied to sensory neurons stripped of plasmalemma and soluble cytosol components by osmotic shock (60 s in distilled water). Stripped nuclei appeared structurally intact and stained with ethidium bromide, indicating retention of chromatin. Measured parameters ($K_d = 771 \pm 131$ nM, $I_{\text{sat}}/I_{\text{free}} = 26 \pm 8$, 7 nuclei) were applied to sensory neuron nuclear measurements. To confirm that the confocal microscope rejected out-of-focus fluorescence from the dye in the bathing medium, 100 μM Fluo-3 was added to the medium bathing intact cells. Signal recorded from the cell interior was $5 \pm 1\%$ ($n = 10$) of the signal recorded from the bathing medium.

METHODS. Patch pipettes contained 140 mM CsCl_2 , 30 mM tetraethylammonium chloride, 7.5 mM HEPES, 5 mM Mg-ATP, 1 mM MgCl_2 and 0.1 mM leupeptin plus fluorescent indicator. External solution was 120 mM NaCl, 5.5 mM KCl, 1.0 mM CsCl_2 , 1 mM MgCl_2 , 1.8 mM CaCl_2 , 25 mM glucose, 20 mM tetraethylammonium chloride, 1 μM tetrodotoxin and 20 mM Tris, pH 7.3. Under these conditions all the recorded inward current is through Ca^{2+} channels²⁴.

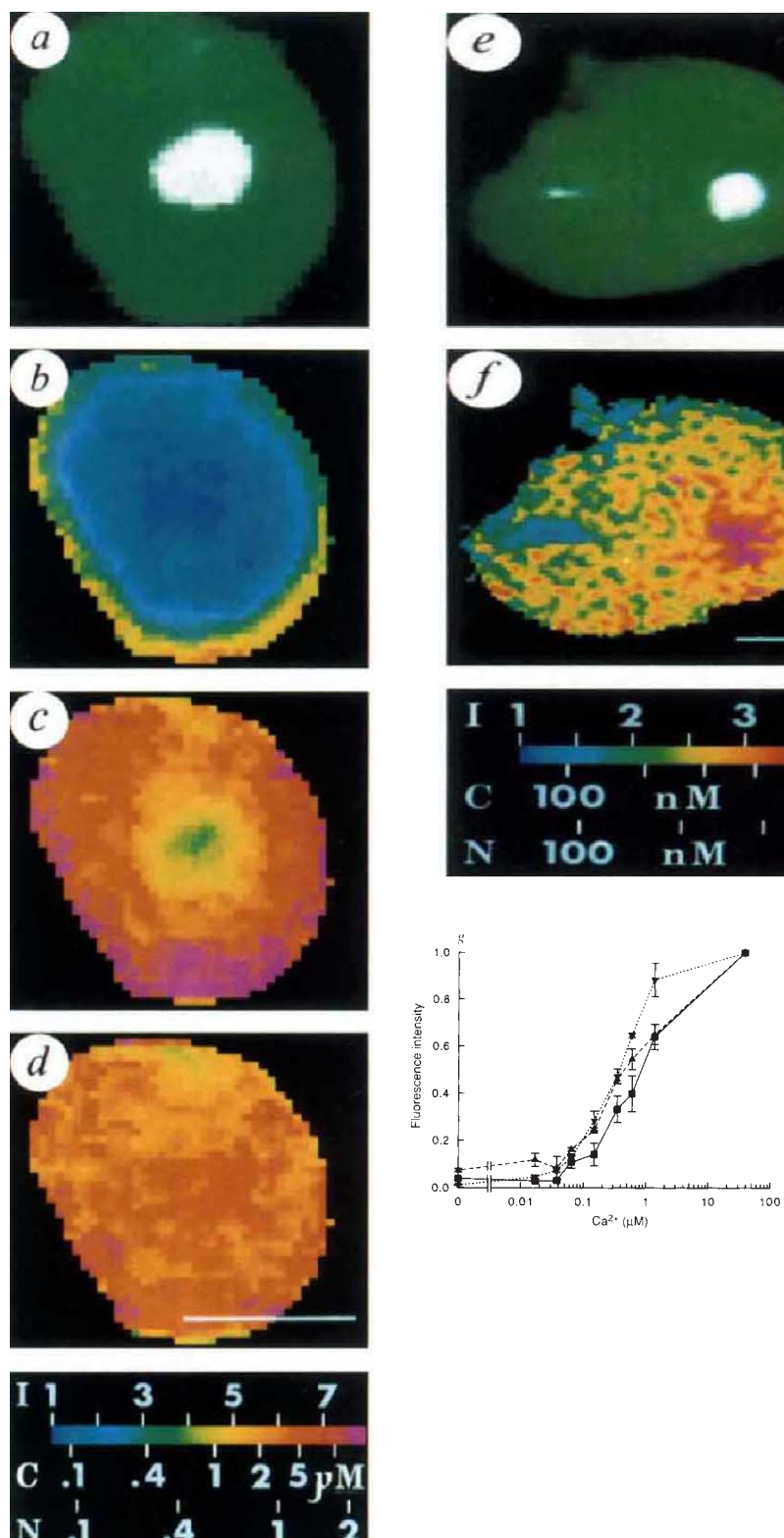
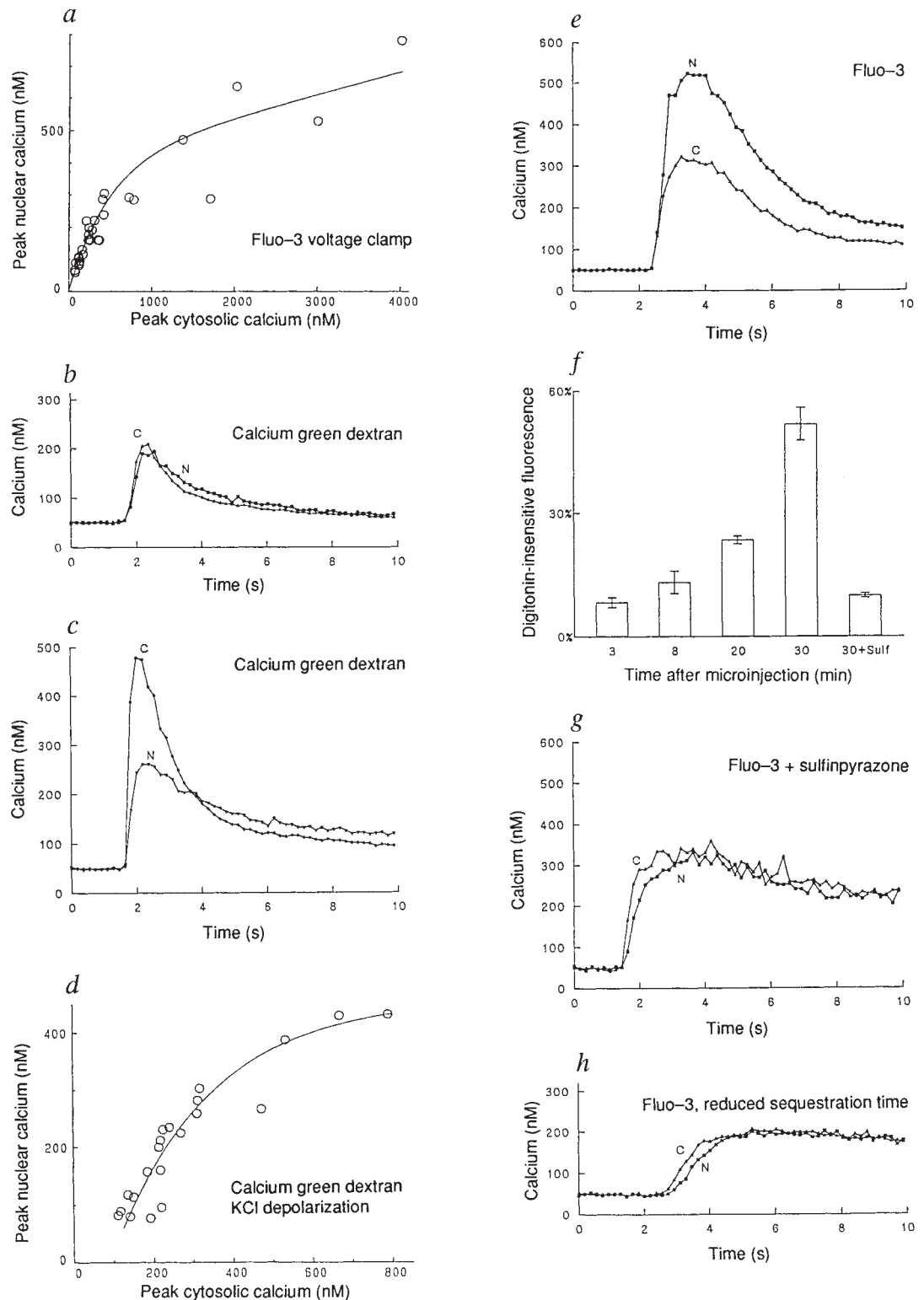


FIG. 4 Nuclear Ca^{2+} dynamics and the amplification artefact. *a*, Nuclear Ca^{2+} changes during patch-clamp depolarization are progressively more attenuated at higher Ca^{2+} . Each circle represents one experiment in which a sensory neuron was depolarized for 1 s or more to one of a range of voltages. The maximum nuclear Ca^{2+} achieved is plotted against the maximum cytosolic Ca^{2+} achieved. To test that nuclear protection is not an artefact of the algorithm used to convert from fluorescence to Ca^{2+} , we examined the raw fluorescence data. The ratio of the fractional fluorescence change in the nucleus to the fractional change in the cytosol (FFCR) is a measure of how faithfully nuclear fluorescence changes follow cytosolic changes. When cytosolic fluorescence increased less than 2.6-fold ($\equiv [\text{Ca}^{2+}]_i < 300 \text{ nM}$), $\text{FFCR} = 0.94 \pm 0.02$, $n = 16$; when $I/I_0 > 2.6$, FFCR was significantly ($P < 1\%$) smaller at 0.82 ± 0.04 , $n = 13$. Placing the small/large ΔCa^{2+} cutoff at $I/I_0 = 3.5$ ($\equiv [\text{Ca}^{2+}]_i = 450 \text{ nM}$) made the difference more dramatic: $\text{FFCR}_{\text{small change}} = 0.92 \pm 0.02$, $n = 22$; $\text{FFCR}_{\text{large change}} = 0.76 \pm 0.04$, $n = 7$, $P < 0.1\%$. *b, c*, Typical Ca^{2+} changes elicited by potassium depolarization ($[\text{K}^+]_{\text{out}}$ increased to 125.5 mM by complete replacement of Na^+) in sensory neurons injected with dextran-conjugated Calcium Green. Where the cytosolic Ca^{2+} change is small (*b*) nuclear Ca^{2+} follows, but where the Ca^{2+} change is large (*c*) the nuclear change is attenuated. Calcium was calculated using a measured $I_{\text{sat}}/I_{\text{free}}$ of 14.0 and K_d of 287 nM . *d*, Scatter plot where each point represents an experiment such as those shown in *b* and *c*. As in patch-clamped cells, nuclear Ca^{2+} changes are progressively more attenuated at higher cytosolic Ca^{2+} . *e*, Apparent nuclear Ca^{2+} amplification in a sensory neuron potassium-depolarized 30 min after injection of Fluo-3. In 10 cells, calculated cytosolic and nuclear Ca^{2+} rose to maxima of $231 \pm 55 \text{ nM}$ and $343 \pm 62 \text{ nM}$, respectively. *f*, Progressive sequestration of Fluo-3 in a non-cytosolic pool. At indicated times after Fluo-3 injection, digitonin was added to $10 \mu\text{M}$ and the cells (at least five at each time point) were incubated for 30 min. For each cell, fluorescence remaining was calculated as a fraction of fluorescence before digitonin. Sulf: sulfinpyrazone²⁰



($250 \mu\text{M}$) present throughout. Sulfinpyrazone has no effect on resting Ca^{2+} (measured with Fura-2 dextran, $[\text{Ca}^{2+}]_{\text{control}} (n = 8) - [\text{Ca}^{2+}]_{\text{sulfinpyrazone}} (n = 13) = 9 \pm 23 \text{ nM}$). *g*, As *e*, but sulfinpyrazone was present throughout and eliminated the apparent nuclear Ca^{2+} amplification. In nine cells, cytosol and nuclear Ca^{2+} rose to maxima of $503 \pm 142 \text{ nM}$ and $469 \pm 154 \text{ nM}$, respectively. *h*, As *e*, but depolarization followed 3 min after dye injection. In five cells cytosol and nuclear Ca^{2+} rose to maxima of $615 \pm 222 \text{ nM}$ and $433 \pm 125 \text{ nM}$, respectively.

entially activated in the cytosol and nucleus. We propose that such enzymes will be activated in the cytosol by brief cytosolic Ca^{2+} transients, but will remain largely inactive in the nucleus, where the Ca^{2+} change is attenuated. Such enzymes will be activated in the nucleus only after a long-lived cytosolic Ca^{2+} change. The pattern of calmodulin activation in neurons is consistent with this scheme: brief depolarization activates cytosolic calmodulin²¹, but calmodulin-dependent transcription of the *c-fos* gene¹ occurs only after intense synaptic stimulation or pathological cell depolarization^{22,23}. □

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Doughnut-shaped structure of a bacterial muramidase revealed by X-ray crystallography

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THE integrity of the bacterial cell wall depends on the balanced action of several peptidoglycan (murein) synthesizing and degrading enzymes^{1,2}. Penicillin inhibits the enzymes responsible for peptide crosslinks in the peptidoglycan polymer³. Enzymes that act solely on the glycosidic bonds are insensitive to this antibiotic, thus offering a target for the design of antibiotics distinct from the β -lactams. Here we report the X-ray structure of the periplasmic soluble lytic transglycosylase (SLT; *M*, 70,000) from *Escherichia coli*. This unique bacterial exomuramidase cleaves the β -1,4-glycosidic bonds of peptidoglycan to produce small 1,6-anhydromuropeptides^{4–6}. The structure of SLT reveals a 'superhelical' ring of α -helices with a separate domain on top which resembles the fold of lysozyme. Site-directed mutagenesis and a crystallographic inhibitor-binding study confirmed that the lysozyme-like domain contains the active site of SLT.

Details of the structure determination are shown in Table 1. The unusually shaped SLT molecule is built up of three domains, which are all very rich in α -helices (Fig. 1). In total, about 63% of all amino-acid residues are in α -helices, the remaining residues being mostly in the helix-connecting turns and loops. The N-terminal domain (residues 1–360) contains 22 α -helices packed in an extended, U-shaped conformation. This U-domain is connected by a long loop of ~20 residues to a small linker domain (residues 381–450) of four α -helices. Together these first two domains form a closed ring with a large central hole (Fig. 1a, c).

The C-terminal domain (C-domain; residues 451–618) is packed on top of this ring (Fig. 1b), interacting with part of the linker domain and the C-terminal region of the U-domain. It has a globular structure, containing nine α -helices, but also includes a very irregular sheet of three small antiparallel β -strands. Overall, the SLT molecule can be described as an asymmetrically shaped doughnut with dimensions of $\sim 85 \times 75 \times 55$ Å. The central hole has a diameter varying from 25 to 35 Å.

The α -helices in the ring-shaped U and linker domains form a double-layered right-handed superhelix. Helices in either layer of this superhelix run parallel to each other, but antiparallel to the helices of the other layer (Fig. 1c). The exception is helix H12, which is inserted as an antiparallel helix in the outer layer, permitting the curve in the U-domain. The helix-to-helix packing in the first two domains of SLT is distinct from that observed in four- α -helix bundles, for which structurally adjacent helix pairs are all antiparallel⁷. This superhelix of helices is not unique, however, having first been observed in the crystal structure of the lipovitellin-phosvitin complex from lamprey yolk⁸. Stabilization is provided by the burying of hydrophobic residues at the helical interfaces and by specific main chain-side chain and side chain-side chain interactions between residues of adjacent helices (Fig. 1d). There is a disulphide bridge between residues 106 and 139 connecting helices H7 and H9. In addition, proline residues are probably important for tightening of the U-domain, especially in the first half where nearly every helix-connecting loop contains one or two of these residues. One proline residue is located in the middle of helix H15, which shows a pronounced kink. Similar kinks in α -helices, caused by internal prolines, have been noted in a number of other proteins^{9,10}.

The C-domain of SLT resembles the fold of lysozyme. Figure 2a and b shows a superposition of the C-domain of SLT on hen egg-white lysozyme¹¹ (HEWL) and lysozyme from bacteriophage T4 (T4L; ref. 12). The structural similarity is remarkable in view of the absence of any significant sequence homology. We showed that the active site of SLT is located in the C-domain by difference Fourier analysis of an SLT-inhibitor complex at 3.5 Å resolution (Fig. 3b, c). Although a more detailed analysis of the SLT-inhibitor complex awaits data at higher resolution, preliminary results confirm the similarity with the lysozymes. The glucosaminyl part of the inhibitor is bound at a position similar to subsite C in the active sites of HEWL and T4L, whereas the proline part is bound close to Glu 478 occupying a region analogous to subsite D in the lysozymes. Glu 478 in SLT most probably functions as the 'catalytic' acid. In the structure alignment it coincides with both the 'catalytic' glutamic acid of HEWL (Glu 35) and that of T4L (Glu 11). Moreover, mutation of this residue to a glutamine gave a completely inactive enzyme,

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in line with a role as protein donor in a lysozyme-like reaction mechanism¹³. In contrast, there is no residue in the β -sheet of SLT that could take up the role of the catalytic aspartate of the lysozymes. Instead, coming from the upper lobe of the C-domain, the carboxylate from Glu 583 is close to the presumed reaction centre (Fig. 2). Its negative charge is, however, not

critical, as a E583Q (Glu $\rightarrow$ Gln) mutant still has considerable catalytic activity. But it may be argued that there should be no strict requirement for a second carboxylate group in the catalytic mechanism of SLT. In SLT the intramolecular C6-hydroxyl group of saccharide D, rather than a water molecule, appears to be the nucleophile that attacks the C1 carbon of a possible

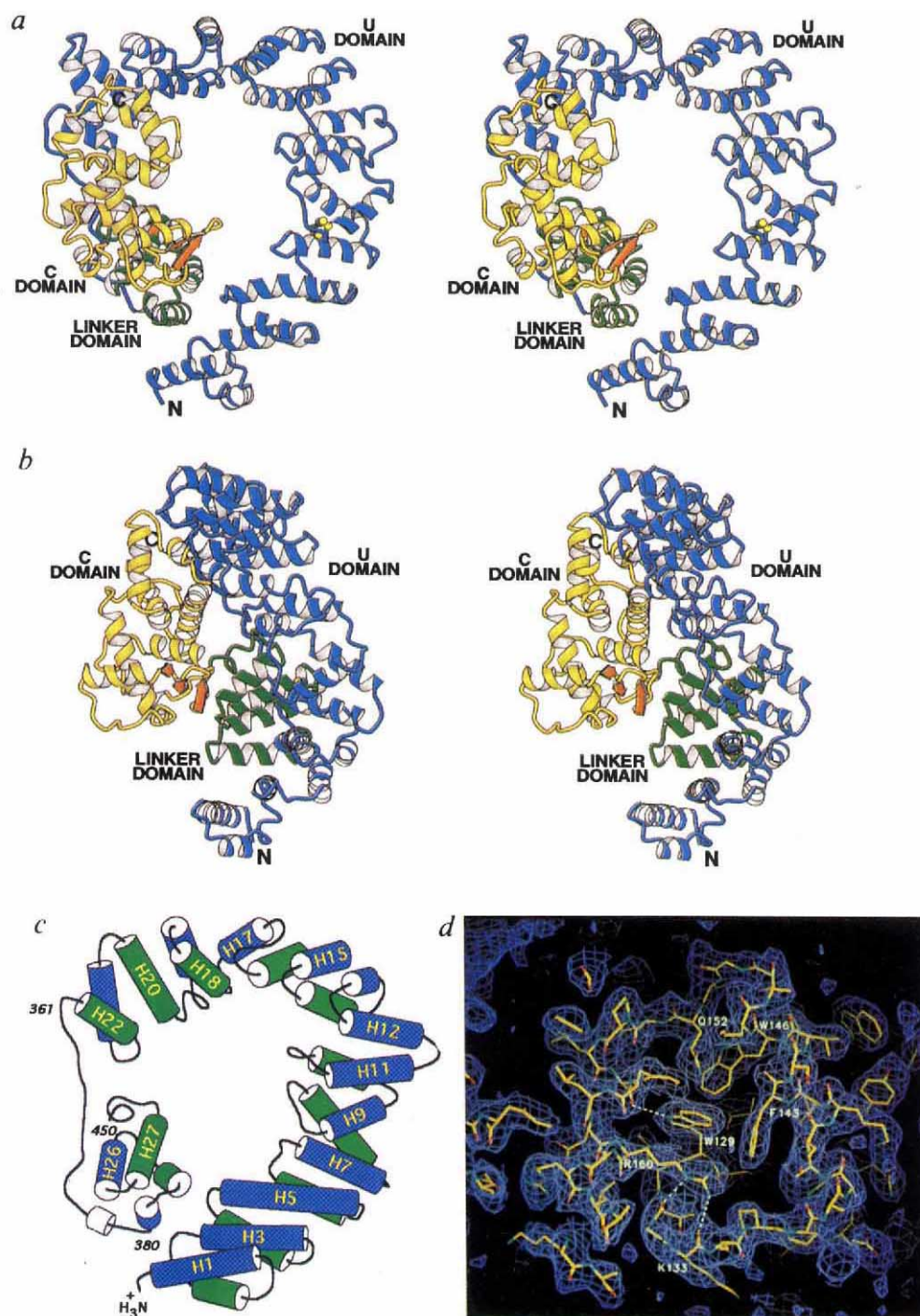


FIG. 1 *a*, A ribbon stereo representation of the model of SLT generated using the program MOLSCRIPT²⁴. The colour assignment is as follows: blue, the N-terminal or U-domain; green, the linker domain; yellow, the C-terminal or C-domain. The N terminus of the polypeptide chain is located at the lower end of the U-domain. The C terminus is located at the top part of the C-domain near the U-C domain interface. Also shown are the atoms of the single disulphide bridge in SLT formed by residues 106 and 139 in helices H7 and H9 of the U-domain. *b*, Edgewise view of the SLT doughnut, showing the C-domain located on top of the helical ring. The active site of SLT is located in the groove of the C-domain

flanked by the three-stranded β -sheet. *c*, Schematic representation of the U and linker domains of SLT illustrating the double-layered 'super-helical' packing of the α -helices. The two layers are identified by different colours of the helices. Residues 361 to 380 form an extended interdomain loop that is wrapped around part of the C-domain in the complete SLT molecule. *d*, A 2.7 Å ($2F_o - F_c$) electron density map calculated with refined model phases and contoured at 1σ , displaying part of the interface of helices H8, H9 and H10 (residues 129 to 165). Possible hydrogen-bond contacts between side-chain and main-chain atoms are indicated by dashed lines.

FIG. 2 Backbone comparison of the C-domain of SLT with lysozyme from hen egg-white (a) and lysozyme from bacteriophage T4 (b) using the Rossmann-Argos alignment method²⁵. The α -carbon backbone of the C-domain of SLT is represented by dark solid spheres connected by thick lines, the backbone of the lysozymes by open spheres and thin lines. Residue numbers refer to the SLT sequence. The overall structure of the C-domain clearly shows most of the features common to the lysozyme fold: it is ellipsoidal in shape with two lobes linked by a long α -helix. A deep groove separates the two lobes. The lower N-terminal lobe includes some helices and a very irregular sheet of three small, antiparallel β -strands. The upper C-terminal lobe is predominantly helical, built up of five different α -helices. Unique features of the SLT C-domain that have no counterpart in either of the two lysozymes include the region between the β -sheet and the longer linker helix, and the last regions of the C-terminal lobe. Also shown are the side chains of Glu 478 and Glu 583 in SLT (dark solid atoms) compared with those of the catalytic residues in the lysozymes (E35/D52 and E11/D20 for HEWL and T4L, respectively). Coordinates for the lysozymes were obtained from the Protein Data Bank²⁶.

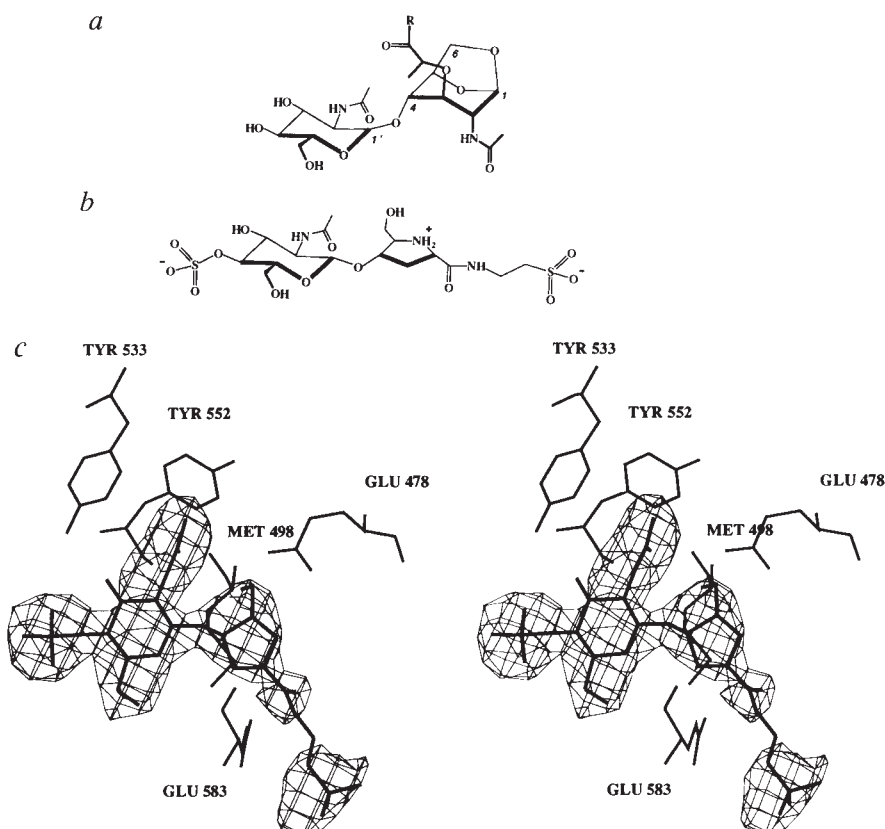
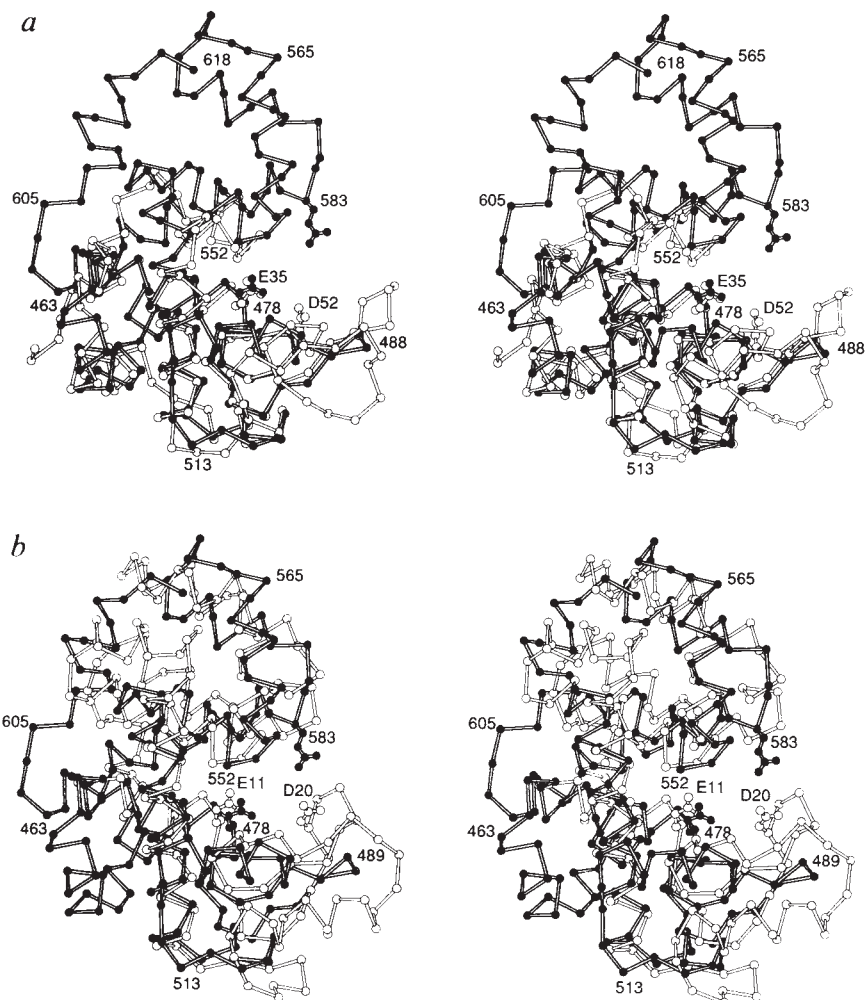


FIG. 3 a, Chemical structure of the mucopeptide product of the reaction catalysed by SLT. The reaction product consists of a disaccharide of N-acetylglucosamine and N-acetylmuramic acid with an intramolecular anhydro-bond between C₁ and C₆. The C₃-lactyl group of the muramic acid is linked to an oligopeptide (R), consisting of L-alanine, D-glutamic acid, meso-diaminopimelic acid and D-alanine residues. The peptide may be crosslinked to that of another disaccharide forming dimeric mucopeptides⁵. b, Structure of bulgecin A. This compound is a bacterial metabolite produced by *Pseudomonas acidophila* and *mesoacidophila*, which has bulge-inducing and lysis-enhancing activity when used in combination with β -lactam antibiotics²⁷. Recently it has been found that SLT is inhibited by bulgecin A²⁸. c, Stereographic view of a 3.5-Å ($F_o - F_c$)exp($i\alpha_c$) difference electron density map of the SLT-bulgecin complex, contoured at 1.5 σ , showing the bulgecin A inhibitor bound in the active site of SLT (F_o represents the observed structure factor amplitudes for the SLT-bulgecin complex, F_c and α_c are calculated structure factor amplitudes and phases from the refined, uncomplexed model). Residues in the active site of SLT that might interact with bulgecin A are indicated. The inhibitor was soaked into the crystals of native SLT for 2 days (7 mM inhibitor in a solution of 40% ammonium sulphate in 0.1 M sodium acetate, pH 5.0). Diffraction data to 3.3 Å resolution were collected on a FAST area detector and merged using programs of the Groningen BIOMOL crystallographic package ($R_{\text{merge}} = 8.1\%$).

TABLE 1 Data collection and phasing statistics

	Native-I	Native-II	KAuCl ₄ -I	KAuCl ₄ -II	Pt(ethylene) (NH ₃) ₂ Cl ₂
Data collection					
Resolution (Å)	3.1	2.7	3.1	3.0	3.1
Observations (no.)	42,639	132,289	23,051	16,287	24,639
Unique reflections (no.)	15,409	32,225	11,062	8,232	12,616
Completeness (%)	87	96	61	45	71
R_{merge}^*	0.052	0.072	0.080	0.110	0.044
$R_{\text{deriv}}^\dagger$			0.17	0.22	0.12
Concentration of HA (mM) [‡]			2	2	0.5
Phasing statistics					
Resolution included (Å)			3.3	3.5	3.3
Heavy-atom sites			3	3	5
Occupancies O_r/O_a [§]			36.1/4.8	28.7/6.1	21.4/3.0
$R_{\text{Cullis}}^{\parallel}$			0.57	0.69	0.62
Phasing power [*]			2.2	1.0	1.9

Native SLT was prepared and crystallized as described^{6,17}. The spacegroup is $P2_12_12_1$ with cell dimensions $a = 80.8$ Å, $b = 88.4$ Å and $c = 132.1$ Å. Native and derivative data used in isomorphous replacement were measured on a FAST area detector (Enraf-Nonius, Delft, The Netherlands) connected to an Elliot GX-21 rotating anode generator, and reduced using the programs MADNES¹⁸, XDS¹⁹ and software of the Groningen BIOMOL package. For the KAuCl₄ derivative, an additional data set (KAuCl₄-II) was collected on the FAST system of the Brookhaven National Laboratory X12C beamline at the NSLS, Upton, NY. Native data to 2.7 Å were collected on the MAR Research image plate system at the synchrotron beamline X31 of the EMBL Outstation at DESY, Hamburg. Evaluation and reduction of these data were carried out using modified versions of the MOSCO programs (Hamburg, Germany) and programs of the CCP4 suite (Daresbury, UK). Heavy-atom parameters were refined with PHARE²⁰. A map was calculated to 3.3 Å which had a mean figure-of-merit of 0.60 for 10,396 reflections between 35.0 and 3.3 Å resolution. The map was improved by solvent flattening and an initial protein model was fitted to the density using FRODO²¹ and O²². The model was gradually improved by energy minimization using X-PLOR²³ alternated with model-building sessions in maps calculated with combined phases. A few rounds of simulated annealing refinement improved the model further. In the last stages of refinement, some water molecules were added, based on peaks in ($F_o - F_c$) difference Fourier maps higher than 3σ and requiring at least two favourable hydrogen-bonding interactions with protein atoms. The present model contains all 618 residues and 22 water molecules. No breaks in the electron density were observed at the 1σ level for the entire polypeptide backbone except at residue 371, which forms part of a long extended loop connecting the first two domains. The current R -factor for 22,643 reflections between 8.0 and 2.7 Å resolution ($F \geq 2\sigma(F)$) is 22.8% after a restrained B -factor refinement. Root-mean-square deviations from ideality in bond lengths and angles are 0.019 Å and 3.6° , respectively.

* $R_{\text{merge}} = \sum_h \sum_i |I_{(h,i)} - \langle I \rangle_{(h)}| / \sum_h \sum_i \langle I \rangle_{(h)}$; $I_{(h,i)}$ is the scaled intensity of the i th observation of reflection h and $\langle I \rangle_{(h)}$ is the mean value.

† $R_{\text{deriv}} = \sum_h |F_{\text{PH}} - F_{\text{P}}| / \sum_h F_{\text{P}}$; F_{PH} and F_{P} are the structure factor amplitudes for native and derivative data.

‡ For data collection and soaking experiments involving heavy atoms (HAs), crystals of SLT were transferred to a stabilizing solution of 40% saturated ammonium sulphate in 0.1 M sodium acetate buffer, pH 5.0.

§ O_r and O_a represent real and anomalous occupancy of the strongest heavy-atom site in arbitrary units, proportional to the number of electrons.

|| $R_{\text{Cullis}} = \sum_h |F_{\text{PH,obs}} \pm F_{\text{P,obs}} - F_{\text{H,calc}}| / \sum_h |F_{\text{PH,obs}} \pm F_{\text{P,obs}}|$; for centric reflections only.

* Phasing power = $[\sum_h |F_{\text{H,calc}}|^2 / \sum_h (|F_{\text{PH,obs}}| - |F_{\text{H,calc}}|)^2]^{1/2}$.

oxocarbenium intermediate (Fig. 3a). Such an intramolecular attack would probably require a shorter lifetime of the oxocarbenium intermediate, and thus less stabilization, as the reaction is no longer dependent on the diffusion-controlled replacement of the leaving group with a water molecule^{14,15}.

The crystal structure of SLT will be used for the design of specific inhibitors that might form a basis for the development of novel types of antibiotics. Meanwhile, further research is needed to obtain more information about the mechanism of

peptidoglycan binding and cleavage. Most intriguing is the question of the function of the U and linker domains. They might be needed to enhance substrate affinity by providing additional murein-binding sites. In addition, the central hole could impose exo-enzymatic activity¹⁶ upon the lysozyme-like C-domain: indeed, the active-site groove in the C-domain is oriented in such a way that a glycan strand bound with its GlcNAc end towards site A would run with its other end through the central hole, close to the surface of the linker domain. □

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DNA recognition by β -sheets in the Arc repressor-operator crystal structure

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TRANSCRIPTION of the *ant* gene during lytic growth of bacteriophage P22 (ref. 1) is regulated by the cooperative binding of two Arc repressor dimers to a 21-base-pair operator site^{2,3}. Here we report the co-crystal structure of this Arc tetramer-operator complex at 2.6 Å resolution. As expected from genetic⁴⁻⁶ and structural studies⁷ and from the co-crystal structure of the homologous *Escherichia coli* MetJ repressor⁸, each Arc dimer uses an antiparallel β -sheet to recognize bases in the major groove. However, the Arc and MetJ complexes differ in several important ways: the β -sheet-DNA interactions of Arc are far less symmetrical; DNA binding by Arc is accompanied by important conformational changes in the β -sheet; and Arc uses a different part of its protein surface for dimer-dimer interactions.

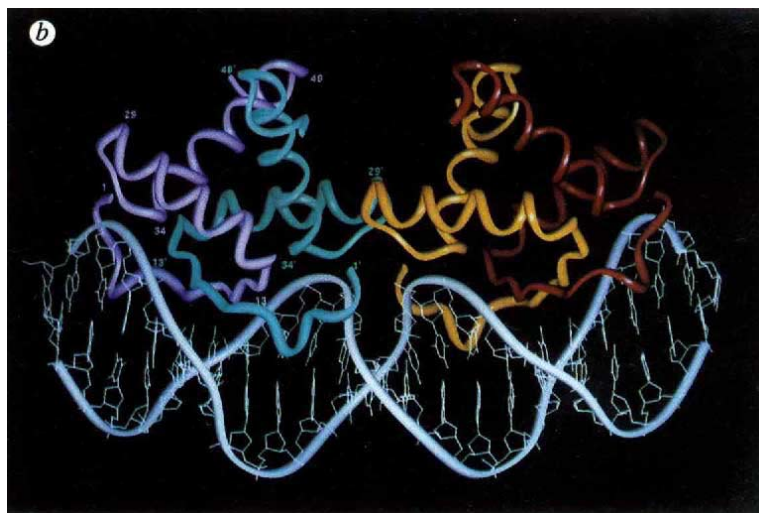
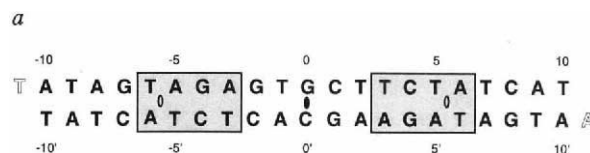
Arc was co-crystallized with DNA containing the wild-type operator (Fig. 1a), and the structure was solved at 2.6 Å resolution using single isomorphous replacement/anomalous scattering (Fig. 1 legend). The final model has very good geometry and a crystallographic R-factor of 22.5% for all reflections from 21 to 2.6 Å. Moreover, the general features of the protein-

DNA complex are consistent with biochemical and genetic studies^{2-6,9-11}. For example, mutations in the TAGA boxes (Fig. 1a) are extremely deleterious to binding⁹, and Arc makes contact with each of these base pairs (bp). As shown in Fig. 1b, each Arc dimer binds to half of the 21-bp operator and the two halves of the complex are very similar (0.5 Å r.m.s. deviation for Ca and phosphorus atoms). Hence, in describing the structure, we focus on the interactions of a dimer with a half-site.

The Arc dimer consists of identical, intertwined monomers of 53 residues: a two-stranded β -sheet is formed by antiparallel pairing of residues 8-14 from each monomer, and α -helices are formed by residues 15-30 (helix A) and 32-48 (helix B). The six amino-terminal residues, which are disordered in the absence of DNA⁷, form tandem reverse turns that are stabilized by interactions with the DNA and with the body of the protein. With the exception of these N-terminal residues and some changes in the β -sheet (see below), the structure of the DNA-bound dimer is very similar to the NMR structure⁷ and crystal structure (C. Kissinger *et al.*, manuscript in preparation) of the unbound dimer.

The β -sheet of the dimer lies flat in the centre of the major groove (Fig. 2a), where Gln 9, Asn 11 and Arg 13 from one β -strand and Gln 9', Asn 11' and Arg 13' from the other β -strand make an extensive set of base contacts (Fig. 2b). Five of these six side chains interact with the first three base pairs of the TAGA box and also hydrogen-bond with each other (Fig. 2b) to form an intricate network at the protein-DNA interface. In addition, conformational adjustments in the β -sheet seem to be important in the recognition process. In the unbound protein, the Phe 10 and Phe 10' side chains are buried in the hydrophobic core, but each side chain swings out and packs between adjacent, unesterified, phosphate oxygens in the complex. At the same

FIG. 1 a, DNA used for co-crystallization. The operator sequence is shown in black letters and the TAGA sequence of each half-site is shaded. The tetramer and dimer pseudo-symmetry axes are indicated by filled and open ovals, respectively. b, The Arc-operator complex. The DNA atoms and backbone ribbon trace are white. The Ca-ribbon traces of Arc monomers in the right dimer are yellow and red, those in the left dimer are blue and purple. Residues in the 'yellow' and 'blue' monomers are marked by primes in the text. For reference, the positions of residues 1, 13, 29, 34 and 48 are marked in one dimer. METHODS. Crystals in space group $P2_1$ ($a=65.7$ Å, $b=56.7$ Å, $c=53.8$ Å, $\beta=107^\circ$) were grown by vapour diffusion with hanging drops containing 1 μ l 0.58 mM Arc tetramer, 0.93 mM DNA duplex, 30 mM bis-Tris-propane-Cl (pH 7.4), 1 mM Na₂S₂O₅, and 1 μ l well solution containing 15% PEG400 and 100 mM MgCl₂. The asymmetric unit contains one tetramer-operator complex. Data were collected on a Rigaku R-Axis IIC detector and reduced using DENZO and SCALEPACK (Z. Otwinowski). Native data to 2.6 Å resolution were collected from two crystals ($R_{\text{sym}}=11.4\%$ for merging 161,619 observations to 11,253 unique reflections; 95% complete). Derivative data to 2.8 Å were collected from one crystal containing 5-iodouridine at operator positions -9 and +9 ($R_{\text{sym}}=11.9\%$ for merging 137,747 observations to 9,335 unique reflections; 99% complete), giving a phasing power of 1.45 and a mean isomorphous difference in intensities of 25%. Heavy-atom parameters were refined with REFIN¹⁴ and SIRAS phases (figure of merit 0.58 from 21 to 2.8 Å) were calculated with PHARE¹⁴. The initial map revealed several base pairs, most of the DNA phosphates and ~80% of the protein main chain. B-DNA and the crystal structure of the Arc dimer were used as guides during initial model building and fitting of electron density using FRODO¹⁵. Most refinement was done using X-PLOR^{16,17} and the model was checked and rebuilt using simulated annealing omit maps in the later stages. At the last stage of refinement, tightly restrained individual B-factors and 45 water molecules were included, and conventional



least-squares refinement with TNT¹⁸ was used. The final model includes all the DNA and all but the last few residues of two monomers. The model has r.m.s. deviations of 0.010 Å from ideal bond lengths, 1.51° from ideal bond angles, and 1.99 Å² in B values between bonded atoms. Only one set of Φ , Ψ angles is non-favourable. Helicoidal DNA parameters were calculated using Dials and Windows¹⁹ using a local helix axis calculated using CURVES²⁰.

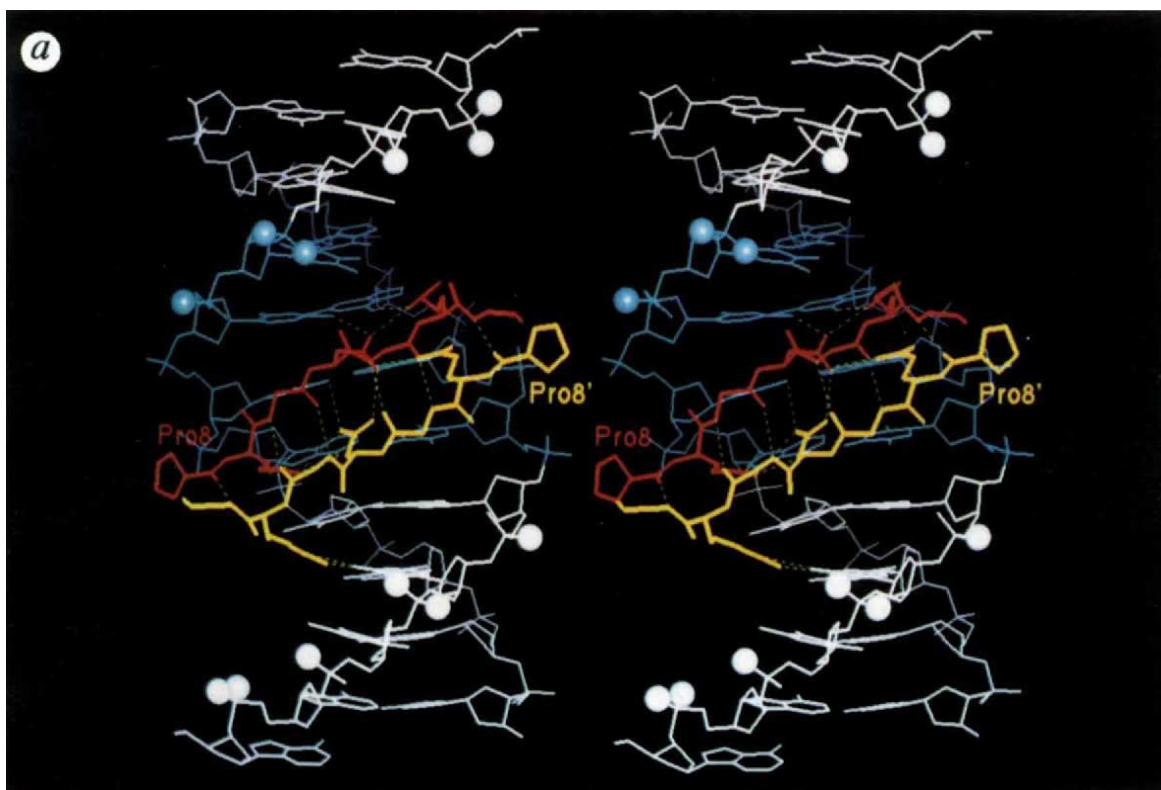
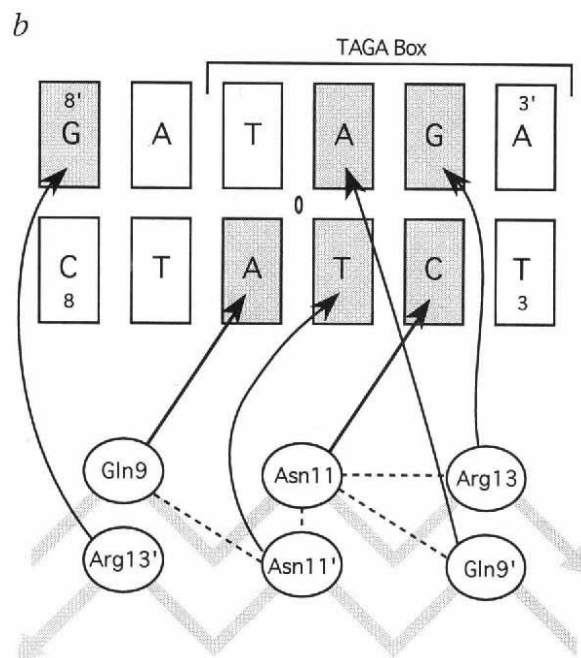


FIG. 2 *a*, Stereo view of the β -sheet in the major groove. The right operator half-site, the backbone of residues 8–14 of the dimer and the side chains of residues 8, 9, 11 and 13 are shown. Atoms from one monomer are shown in red and those from the other in yellow. The DNA is white, except for the TAGA box which is blue. Phosphate oxygens contacted by the Arc dimer are represented as spheres. Hydrogen bonds are shown as green, dashed lines. Pro 8 of each monomer is labelled. *b*, Hydrogen bonds between β -sheet side chains and DNA bases in the right half-site are indicated by arrows, and hydrogen bonds between side chains are indicated by dashed lines. The dimer symmetry axis is indicated by an open oval, and contacted bases are shaded. Gln 9 and Gln 9' accept hydrogen bonds from Asn 11' and Asn 11, respectively. Asn 11 accepts a hydrogen bond from the N ϵ atom of Arg 13 and donates a hydrogen bond to Asn 11'. The same contacts are seen in the left half-site except that Arg 13' makes a contact with an adenine at position –8 rather than a guanine.



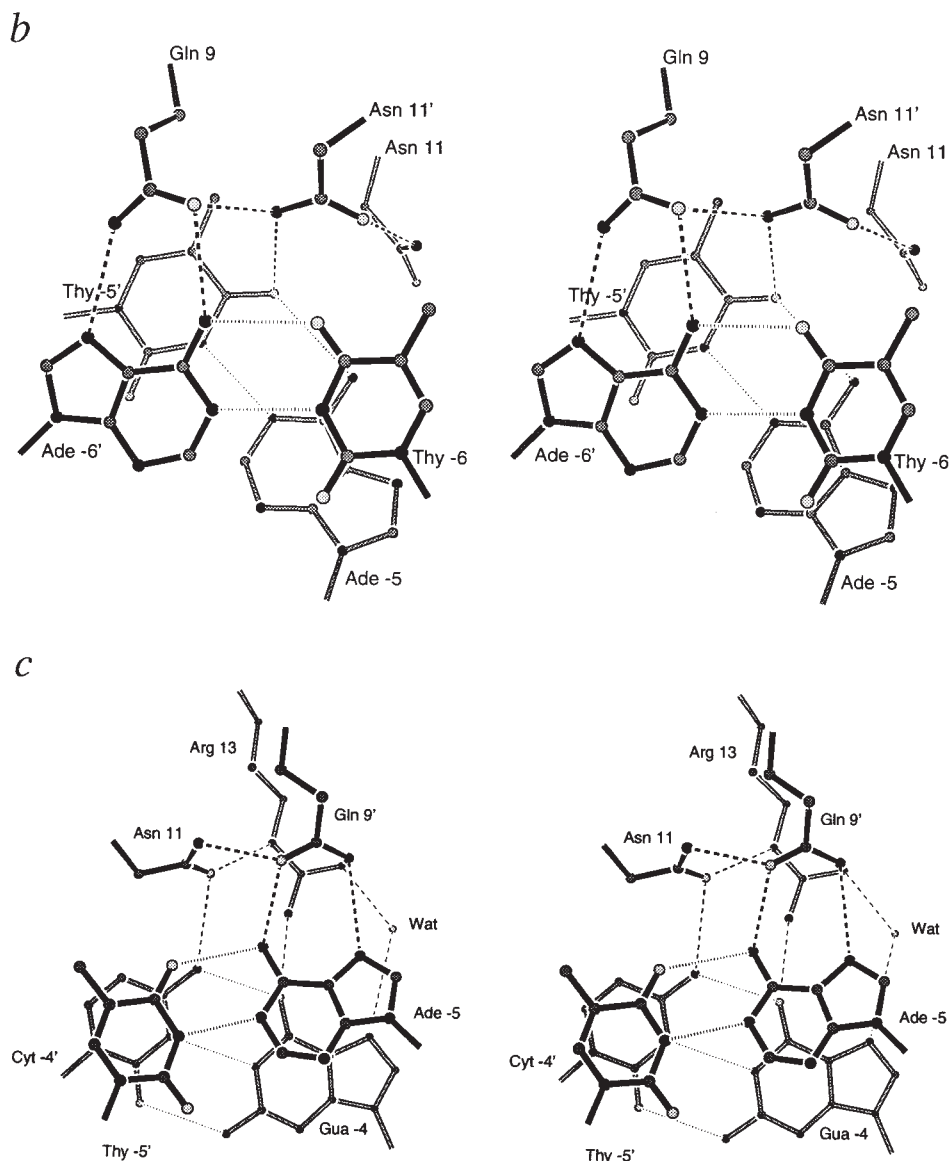
time, the C α atoms of residues 9, 9', 10 and 10' move 1.0–1.7 Å to fill partially the cavities left by the repositioning of the phenyl rings. The main-chain movements of Gln 9 and Gln 9', in turn, help to position the side chains of these residues for base contacts.

Figures 3 and 4 show details of the side-chain–base contacts. The TAGA box contacts in the right half-site include: pairs of hydrogen bonds between Gln 9/Gln 9' and adenines 6 and 5, respectively, single hydrogen bonds between Asn 11 and cytosine 4 and Asn 11' and thymine 5, and one hydrogen bond between Arg 13 and guanine 4 (all the corresponding contacts are also observed in the left half-site, plus an additional, water-mediated hydrogen bond between Arg 13 and guanine –4). There are no hydrogen bonds to the last base pair of the TAGA box, but there are hydrophobic interactions involving the thymine methyl group, Met 4' (in the N-terminal arm) and the aliphatic portion of the Arg 13 side chain. The other thymine methyls in the TAGA box are also buried and make van der Waals contacts with atoms from the β -sheet (Fig. 3). The only hydrogen bonds to bases outside the TAGA box are made by Arg 13', which makes two hydrogen bonds with guanine 8 in the right half-site

(Figs 2*b* and 3) and one hydrogen bond with adenine –8 in the left half-site.

Although Arc is an inherently symmetrical protein dimer, it recognizes a largely asymmetrical base sequence. The symmetry axis of the dimer lies between the first and second base pairs of the TAGA box and is roughly coincident with a symmetry axis that could be defined from the set of phosphate contacts (discussed below). The T·A and A·T base pairs at the first and second positions of the TAGA box are related by the dimer symmetry axis (Figs 1*a* and 2*b*), and indeed, Gln 9 and Gln 9' do make symmetrical contacts with the adenines at these positions. However, there is no further symmetry in the base contacts. This

FIG. 4 Atomic view of contacts between Arc and operator bases. *a*, Electron density for β -strand residues 8–14 from an $|F_o| - |F_c|$ simulated annealing map in which these residues were omitted. For reference, the model for base pairs –4 to –6 and the water-bridging Arg 13 and base –4 are also shown. *b*, Stereo view of contacts in the region of Gln 9. The side chains of Gln 9, Asn 11 and Asn 11' and base pairs –5 and –6 are shown. *c*, Stereo view of contacts in the region of Gln 9'. The side chains of Gln 9', Asn 11 and Arg 13 and base pairs –4 and –5 are shown. In *b* and *c*, bonds near the viewer are darkened. Short dashes indicate hydrogen bonds between bases; all other hydrogen bonds are shown by long dashes.



Arc and MetJ are members of the ribbon-helix-helix family of DNA-binding proteins¹³. Although these proteins use different sets of β -sheet residues to recognize different DNA sequences¹³, comparison of the Arc and MetJ⁸ co-crystal structures shows that the protein dimers dock against DNA in very similar ways (1.5 Å r.m.s. deviation for common Ca and phosphorus atoms). However, MetJ dimers in the co-crystal recognize symmetrical DNA sites whereas Arc dimers do not, and most of the side chains at the corresponding positions of the β -sheets in Arc and MetJ make contact with different operator base positions. Moreover, unlike the β -sheet of Arc, that of MetJ does not undergo conformational changes on DNA binding. Finally, there are major differences in the protein regions used for cooperative contacts because the half-sites in the MetJ and Arc operators have different spacings; DNA-bound MetJ dimers interact along the entire length of helix A, whereas Arc dimers interact using the loop between helices A and B. These comparisons indicate that proteins of this family can adapt to recognize different DNA sequences by changing the chemical identity of β -sheet residues, by adjusting side-chain and main-chain conformations to allow corresponding β -sheet positions to make contact with different base positions, and by using different parts of the protein for cooperative interactions. □

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Oligonucleotide probe design — a new approach

Masato Mitsuhashi, Allan Cooper, Mieko Ogura, Tatsuo Shinagawa, Katsusuke Yano and Toshiaki Hosokawa

The use of oligonucleotides for gene amplification, as diagnostic probes or antisense-based drugs can be optimized by minimizing the possibility of nonspecific hybridization.

Now that oligonucleotides can be easily synthesized, and reporter molecules such as biotin and primary amines incorporated during synthesis without time-consuming procedures, oligonucleotides are becoming increasingly popular as nonisotopic detection probes for Southern and northern blots, point mutation, and *in situ* hybridization¹, as well as immobilized capture probes². Powerful gene amplification methods such as the polymerase chain reaction (PCR)³, and ligase chain reaction⁴ also require oligonucleotides as primers. Another interesting application of oligonucleotides is the blocking of translation-specific genes, and the pharmaceutical industry is now investigating antisense

oligonucleotides as potential therapeutics for some infectious diseases and cancers⁵.

The main obstacle to their effective use is the difficulty of determining specific sequences of oligonucleotides for each application. For example, it is still difficult, even for experienced molecular biologists who have extensive knowledge of the structure and function of the target gene, to design a 20-mer oligonucleotide sequence that hybridizes to only the desired target gene and not to undesired genes.

In addition to providing primer pairs that satisfy user-defined parameters such as length of primers, GC content, length of PCR products, the new design strategy described here provides homology information of all the candidate primers, allowing users to know whether selected primer(s) will cross-hybridize to unrelated genes, or not.

Principle

The new computer program (OligoProbe DesignStation) we have developed⁶ can extract potential probe sequences from every location of the target gene of interest based on a melting temperature (T_m) of probes specified by the researcher (Fig. 1). If the target gene is 1,000 base pairs, for example, the computer extracts approximately 1,000 candidate probes, one starting from each nucleotide base in the target gene (Fig. 1a).

As T_m represents the actual binding strength of hybridization and stringency of assay conditions, two probes with the same T_m , or similar T_m , can provide high sensitivity and selectivity for a pair of PCR primers or sandwich probe assays. More interestingly, once experimental conditions are established with one particular oligonucleotide probe, the same assay conditions can be ap-

plied to other probes for a variety of different target genes. Furthermore, multiple genes can be analysed simultaneously in a single experimental system using multiple oligonucleotide probes. For antisense applications, once an optimal T_m is determined for intracellular physiological conditions, any gene function can be easily modified by antisense oligonucleotides.

Each candidate probe is then analysed for potential hybridization against a gene database, typically GenBank, using an algorithm for fast homology analysis. In a homology calculation for mismatched probes, the program calculates T_m at the longest non-mismatched stretch of nucleotide sequence, followed by mismatches containing longer sequences. The actual T_m is determined as the highest value among calculated T_m . After a 15–30 min analysis on a 486 IBM PC or compatible computer, users can see the name of identical genes and those that cross-hybridize, the position and the estimated T_m for each gene, location of mismatched nucleotides (Fig. 1b), length, content of each nucleotide, maximum energy of hairpin formation and energy of association between identical oligonucleotides (stacking energy) for each candidate probe (Fig. 1c).

In order to quantify the specificity of each probe, the program also calculates the number of cross-hybridizing sequences in the databases with T_m of 4 °C, 8 °C, 12 °C, 16 °C, 20 °C, 24 °C, 28 °C and 32 °C below the value defined by the user. These data can then be exported to a spreadsheet program, such as Microsoft Excel (see table) for further analysis.

Examples

Simple PCR primers. The human interleukin 1- α gene (HUMIL1AR)⁷ was retrieved from GenBank, and probes with a T_m of 54 °C were extracted from every position of this target gene. Then, the candidate probes were analysed for homology against the GenBank primate database as described above. As five IL-1 α mRNAs/cDNAs and one genomic IL-1 α sequence are registered in GenBank under a different name, we chose probes at a common region among six different entries of IL-1 α in order to avoid any sequencing or typographical error during entry (see table). The main criteria used in choosing the probes was their low probability of cross-hybridization. None of the probes chosen should cross-hybridize with

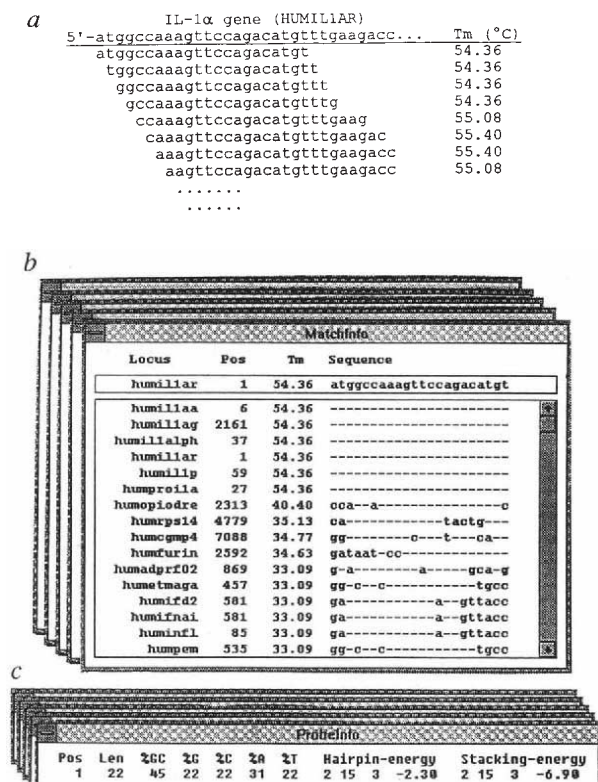


FIG. 1 Principle of the program. a, The program (OligoProbe DesignStation) can extract potential probe sequences from every location of the target gene of interest based on a melting temperature (T_m) (54 °C in this case) of probes specified by the researcher. Then, each candidate probe is analysed for potential homology against a gene database, such as GenBank, using an algorithm for fast homology analysis. The name of genes that cross-hybridize, the position of the each gene, the estimated T_m , and the location of mismatched nucleotides (b), length, content of each nucleotide, hairpin energy and stacking energy (c) are presented for each candidate probe.

Summary of probe selection in a spreadsheet program

Selection criteria		ΔT_m^*							Hairpin >0	Stacking > − 5	Sequence
Position <500	Length <26	0 °C =6	4 °C	12 °C	20 °C =6	24 °C	28 °C	32 °C			
Selection result											
64†	23	6	6	6	6	43	686	4520	2.4	−3.9	AGTTCCTCCATTGATCATCTGTC
65	22	6	6	6	6	31	400	4130	2.4	−3.9	GTTCTCCATTGATCATCTGTC
66	23	6	6	6	6	47	398	5285	2.4	−3.9	TTCTCCATTGATCATCTGTCTC
67	22	6	6	6	6	36	346	4638	2.4	−3.9	TCCTCCATTGATCATCTGTCTC
68	22	6	6	6	6	42	387	5016	3.8	−3.9	CCTCCATTGATCATCTGTCTCT
169	25	6	6	6	6	37	660	3818	2.93	−2.1	TCTGAAACCTCTAAAACATCCAAGC
170	24	6	6	6	6	35	555	3439	2.93	−2.1	CTGAAACCTCTAAAACATCCAAGC
174	25	6	6	6	6	42	593	3233	4.13	−3	AACCTCTAAAACATCCAAGCTTACC
175	24	6	6	6	6	42	535	3024	4.13	−3	ACCTCTAAAACATCCAAGCTTACC
176†	23	6	6	6	6	37	290	2868	4.13	−3	CCTCTAAAACATCCAAGCTTACC
416†	25	6	6	6	6	12	188	1242	1.07	−4.2	CCCTCAATCAAAGTATAATTCTGAGC
417	25	6	6	6	6	6	278	1138	1.07	−4.2	CCTCAATCAAAGTATAATTCTGAGCC
463	25	6	6	6	6	30	784	3950	2	−4.8	GCTGCATTACATAATCTGGATGAAG

*Number of gene in GenBank primate database that could hybridize with the specified probe at the ΔT_m below the user-defined T_m (54 °C). $\Delta T_m=0$ values are the number of identical sequence matches found to the probe

† Selected probes

any other sequences in the GenBank primate database at 20 °C below the T_m . In addition, probes were selected using the conditions of having the hairpin energy greater than 0 kcal mol⁻¹ and stacking energy greater than -5 kcal mol⁻¹ (see table).

The next analysis was undertaken to verify the location of mismatches as shown in Fig. 1b for each of the selected probe candidates because mismatches near the 3' end are

more critical than 5' end for successful PCR results. The final analysis was to determine if two primers would make an optimal pair. Each possible pair of primers was submitted to a two-probe hybridization analysis using the program, and the energy of possible associations between the two primers was then determined. Because of the high concentrations of primers used in PCR, interaction between the two primers can greatly reduce the sensitivity of PCR.

The result of the PCR experiments is shown in Fig. 2, in which PCR products from our primers with cross-hybridization analysis (Figs 2a and b) can be seen from 10,000–100,000 times more diluted template than those of primers without cross-hybridization analysis (Fig 2c). This may be due to efficient hybridization of the primer to the target gene with less false-hybridization to unrelated genes present in the reaction mixture. Additionally, the primers designed by this method displayed a wider range of Mg²⁺ concentrations ranging from 0.5 mM to 3 mM and annealing temperatures between 45 °C and 65 °C (data not shown).

Complex PCR primers. Initially, the target B1 coxsackievirus gene (CXA1G)⁸ was retrieved from GenBank, and an analysis was conducted for a T_m of 54 °C. In order to design PCR primers that will amplify all coxsackieviruses with a minimum chance of cross-hybridization to other viruses and human genes, an analysis was conducted using three different databases, the GenBank primate, GenBank virus, and a database consisting of all the coxsackieviruses, such as A9, A21, B1, B3 and B4.

In the analysis using the coxsackievirus database (Fig. 3a), several probes were found that exhibited homology among five coxsackieviruses. By considering cross-hybridization to human and other virus genes, the secondary structure of probes, 3' GC

content, the size of PCR product, and primer-dimer formation as described in the example above, probes #415 and #608 (# indicates the starting point of each oligonucleotide

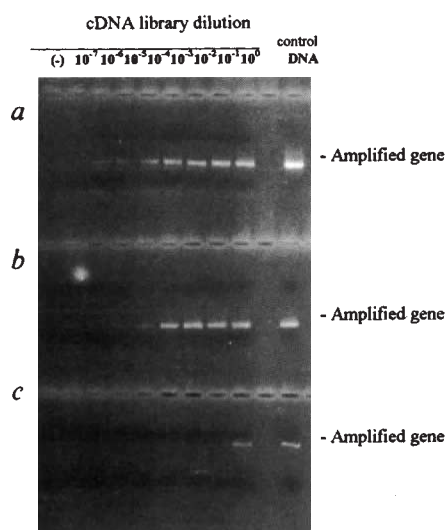


FIG. 2 Results of PCR amplification of human IL-1 α gene. Human leukocyte cDNA library (Clontech, Inc., Palo Alto, California) was diluted by water, and 1 μ l of cDNA was subjected to PCR in a final volume of 10 μ l with 30 cycles of 55 °C annealing for 1.5 min, 72 °C extension for 4 min and 94 °C denature for 1.5 min. PCR was conducted using primers with cross-hybridization analysis (a, #176–#416 and b, #64–#416) or without cross-hybridization analysis (c, #1–#784). # indicates the starting position of each oligonucleotide within the target gene for comparison. PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide.

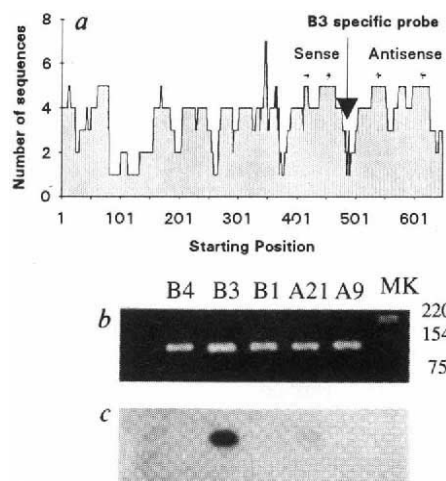


FIG. 3 Summary of PCR primers common for all coxsackieviruses and species-specific PCR primers. a, The number of sequences in the GenBank primate database that will cross-hybridize with the target coxsackievirus type B3 at 16 °C below the T_m . Four horizontal arrows indicate the location of sense and antisense primers and the vertical arrow indicates the location of type B3-specific probe. b, Result of PCR amplification. The culture supernatants of five coxsackieviruses (American Type Tissue Culture Collection, Bethesda, Maryland) were subjected to PCR with #415 and #608 as the first round PCR primers under the identical conditions to that of Fig. 2. Then, 1 μ l of the PCR products was further amplified by the second round PCR with #445 and #543. PCR products were analysed by agarose gel electrophoresis and stained with ethidium bromide. c, Result of Southern blot analysis. The agarose gel as shown in Fig. 3b was blotted onto a nylon membrane and probed with ³²P-labelled type B3-specific oligonucleotide (#485). The probe was labelled with ³²P-ATP using T4 nucleotide kinase.

within the target gene) were chosen as the first round PCR primers, and #445 and #543 as the second round of nested PCR primers, respectively. As shown in Fig. 3b, the target gene was successfully amplified by this nested PCR from all five coxsackieviruses. In order to identify the species of coxsackievirus from the PCR product, a species-specific probe was also designed, as shown in Fig. 3a, in which the lowest point between the two primers exhibited highest specificity. Southern blot analysis proved the specificity of the designed B3-specific probe (Fig. 3c). The specific probes for other types were also successfully designed by the same methods (data not shown). The procedure described here may have application in clinical diagnostics for diseases such as hepatitis, AIDS or genetic disorders.

Discussion

The new design strategy discussed here allows researchers to use gene sequence data deposited in public databases such as GenBank and EMBL directly in the design

of oligonucleotide probes, primers, and antisense-based drugs for various genes of interest. Furthermore, the methodology can also be used in the design of more complex probes, such as cross-species probes and probes to identify undiscovered genes in the same family, by choosing areas of highest consensus within the known members and the lowest chance of cross-hybridization (Fig. 3). Another advantage of this technology is that additional requirements for probe design can be easily implemented and customized using the features of spreadsheet programs to manipulate the data and select for other criteria. Although the gene sequences in these databases are incomplete and therefore cannot yield the entire homology analysis, the methodology described may prove useful in reducing the number of candidate probes used in experiments. □

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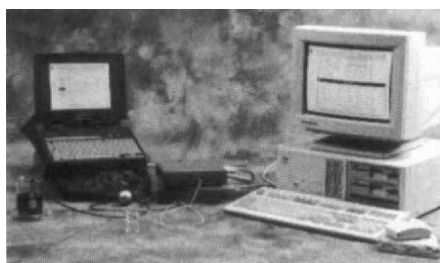
California 92715, USA and Hitachi Chemical Research Center, Inc., Irvine, CA 92715, USA. Allan Cooper is at AGCT, Inc. and DigitalWord, Bellevue Washington 98008, USA. Mieko Ogura is at Hitachi Chemical Research Center, Inc., Tatsuo Shinagawa and Katsusuke Yano are in the Third Department of Internal Medicine, Nagasaki University, School of Medicine, Nagasaki, Japan and Toshiaki Hosokawa is at Hitachi Chemical Company Ltd, Tokyo, Japan. We thank Dennis Coad, David Hyndman, Dr Takashi Ishikawa and Dr Yasushi Ichikawa for their helpful comments. For more information, fill in reader service number 100.

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Software and CD-ROMs

Computer buffs may be interested in this week's selection which includes software developed specifically for the chemist, a program for densitometry and gel analysis and a lab manual on CD-ROM.

EARLIER this month, Strawberry Tree began shipping its DATAshuttle series of peripheral analog input and digital I/O devices, which connect via the parallel port to desktop and portable personal computers (*Reader Service No. 101*). When connecting DATAshuttle, there is no need to open the PC, set board addresses, adjust calibration or



DATAshuttle peripheral device for data acquisition on the PC.

worry about hardware conflicts. In addition to hardware, the company provides Quicklog PC application software at no extra cost. Or for users operating in the Windows environment, the DATAshuttle integrates with the new WorkBench PC for Windows. The DATAshuttle family uses a high-speed parallel 'pass-through' interface providing a port for other devices such as printers and modems. In addition, up to 15 DATAshuttles can be linked together in series to accommodate up to 120 analog inputs and 120 digital I/Os. The DATAshuttle is available in 12-bit and 16-bit models for general-purpose voltage inputs and high accuracy temperature

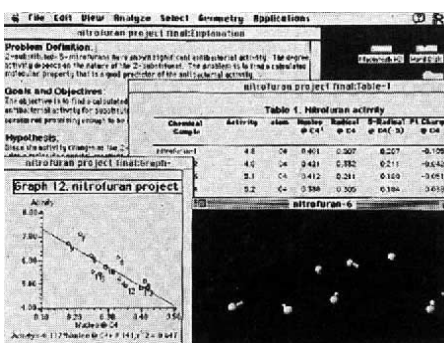
measurements using thermocouples or RTDs. Prices start at US\$995 for the series.

Chemistry

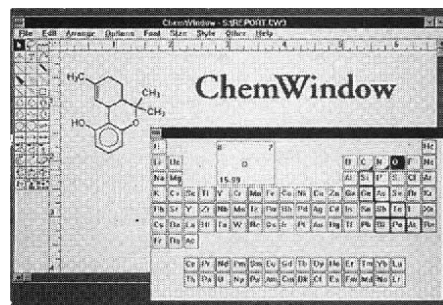
CACHE Scientific is shipping its ProjectLeader software for the bench chemist, which is designed to handle computational experiments in the context of an R & D project — from planning, set-up and execution to analysis and publication (*Reader Service No. 102*). To use ProjectLeader, chemists simply create tables that specify compounds to be analysed, determine properties they wish to estimate and select which methods to use. It organizes and executes research experiments, placing the calculated results back into a table for easy analysis. CACHE Scientific says that it can be used to estimate properties such as solubility, pK_a values, heat of formation, spectra, suscepti-

bility to nucleophilic and electrophilic attack using applications such as MOPAC and ZINDO. Results are presented in a way that enables chemists quickly to try out new ideas and perform analyses such as QSAR (quantitative structure activity relationships) and QSPR (quantitative structure property relationships). The program interfaces with spreadsheet, statistical and presentation applications to simplify archiving, internal reporting and publishing.

SoftShell has updated its ChemIntosh and ChemWindow chemical drawing software (*Reader Service No. 103*). Version 3 is



Nitrofurantoin project — see ProjectLeader.



Software upgrade for ChemWindow.

compatible with most Macintosh and Windows applications and is said to have greatly enhanced the power of previous versions while still remaining easy to use. Among the new features are 'hot' keys for atom labels and groups. Version 3 upgrades are free to those who purchased any of the two pro-

grams after 1 March 1992 and US\$99 for those who purchased before that date. For first-time buyers, the programs cost US\$599.

Flexifit is the new enhancement to Chemical Design's Chem-X **three-dimensional (3-D) database searching system** (*Reader Service No. 104*). It works by allowing molecules from a 3-D database search to 'relax' to fit the search query. It provides an alternative to using conformational searching which, although rigorous, is said to be slow with large, flexible molecules. Both methods rely on storing one set of coordinates and rapidly generating alternative coordinates by rotating about bonds. Chem-X runs on a wide range of platforms including UNIX workstations, DEC, Alpha, PC (DOS and Windows) and Macintosh computers.

■ Imaging

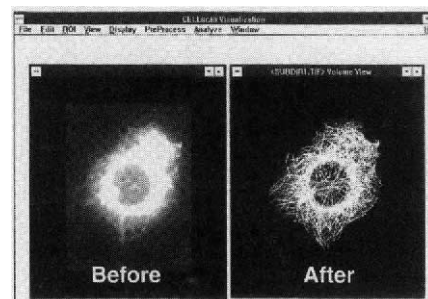
Data Cell's new **MIRAGE image processing and analysis systems** are designed as complete systems for applications in microscopy, biomedical sciences and materials sciences (*Reader Service No. 105*). Three



Data Cell's new MIRAGE image processing systems come in standard, advanced and R & D versions.

systems are offered: standard, advanced and research and development. They comprise a complete system, including both hardware and image processing software, together with image library, database, spreadsheet and report writing facilities. The standard and advanced level systems come with a camera, 486/66 MHz computer with one or two high-resolution monitors and special display cards, 400 MB disk drive and hardware that includes a framegrabber, optional DSP signal processor, image compression card, recursive averaging hardware and optical disk drive. MIRAGE systems offer pixel intensity, point line and area measurements, wall thickness aspect ratio, counting and colour sequence analysis and are supplied with Microsoft Office (which includes Word, Excel and the Powerpoint presentation graphics package). The advanced system also provides a powerful macro facility that allows applications to be developed quickly, and can include enhanced colour analysis, automatic classification of objects and automatic transfer of data to Office or other packages. At the high end, the MIRAGE R & D system is usually supplied with a Sun SPARCstation computer and uses Visilog software.

The **CELLscan image enhancement system** from Scanalytics is designed to provide quantitatively accurate two- (2-D) or three-dimensional (3-D) images of living or fixed cells (*Reader Service No. 106*). The sys-



CELLscan system for high-resolution fluorescence microscopy.

tem's cooled CCD camera and piezoelectric focusing device automatically acquire a through-focus set of images, and the 'exhaustive photon reassignment' algorithms analyse the information to provide 2-D and 3-D images. CELLscan runs on an IBM PC or compatible computers under Microsoft Windows.

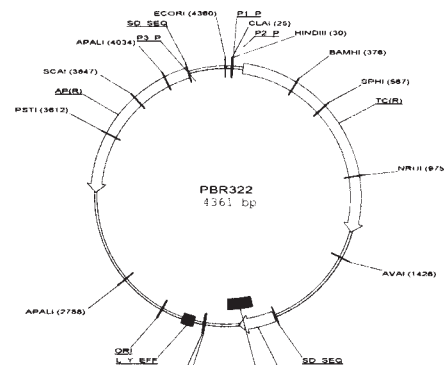
Fullpixelsearch software from Avian Systems allows users to **search and find important regions inside TIFF or PICT images** on any colour-enabled Macintosh computer (*Reader Service No. 107*). The program matches patterns at the pixel level, a function that previously has been available only on mainframe systems, according to the company. It allows users to locate regions of interest with a large degree of control of the search criteria; identify important regions in any image, and define a search based on those regions; save, as separate files, search templates and the location of all matches or finds generated; generate histograms for any selected region of an image and display coordinates and spectral class value for any pixel; and generate a 'neighbour report' for all pixels of any colour enabling the user to quantify the degree of randomness or uniformity of an image. The US\$895 program should be of interest to earth scientists, pathologists and cell biologists, scientific illustrators, astronomers and planetary scientists.

■ Molecular biology

Signal Analytics has introduced new **densitometry and gel analysis software** for the Macintosh called IP Lab Gel (*Reader Service No. 108*). IP Lab Gel's manual drawing tools can outline the bands to analyse, or the global thresholding command can identify multiple bands at once. Users can report density statistics and position information for each band or blot, perform region-of-interest processing, and identify peak positions and values. A plot profile command lets users obtain a plot of the average of the pixel values along each row of a region of interest. The peaks can be marked manually

or with the automatic peak-finding command, and the total area under the peaks can be analysed. The resulting statistics report the integrated intensity, the mean density, and the standard deviation of the density values in each band or blot. Users can also find the peak position and value, and the fraction of total material under each peak. The program reads standard PICT and TIFF files as well as Molecular Dynamics' PhosphorImager data files. The plots and processing results can be exported to popular spreadsheet and word processing programs. Version 1.1 is now shipping and costs US\$750. The requirements are a Macintosh with a floating point processor. IP Lab Gel-LC, which costs US\$600, has the same capabilities as IP Lab Gel but is for use with Macintosh computers without FPU's.

Vector NT from InforMax is a new software system for the **automatic design of genetic molecules** running on any computer that runs Windows 3.1 (*Reader Service*



Vector NT for Windows.

No. 109). Using the US\$995 program, researchers will receive the system's recommendations for optimum cloning steps when constructing recombinants of any size. Users may adjust Vector NT's knowledge base, keeping their own preferences of genetic cloning and manipulation. The system's database is capable of transferring changes in child-parent trees of molecules. The system can perform comprehensive PCR and other standard genetic engineering operations.

■ Electronic books/manuals

The looseleaf **laboratory manual**, Current Protocols, is now available as an interactive electronic media version on CD-ROM from Current Protocols, a joint venture between Greene Publishing Associates and John Wiley (*Reader Service No. 110*). Each looseleaf manual is comprised of hundreds of step-by-step laboratory research methods, recipes for the preparation of reagents, illustrative diagrams, tables and bibliographic references — all cross-referenced. The electronic search and retrieval tools with the CD-ROM version, which includes both Current Protocols in Molecular Biology and Current Protocols in Immunology, NATURE · VOL 367 · 24 FEBRUARY 1994

may be a useful aid to bench scientists who need to access information quickly. Some of the features of the CD-ROM include a graphical user interface that is compatible with both Macintosh or Microsoft Windows platforms, search capabilities based on boolean logic, hyperlinked 'jumps', copy-and-paste capabilities, bookmark and 'sticky note' features for quick recall, and quarterly updates.

MathSoft announces a new **electronic book on electromagnetics** (*Reader Service No. 111*). The US\$49 book, *Theory and Selected Problems of Electromagnetics* from Schaum's Outline Series, provides an online reference to laws governing electromagnetics. It is used with MathSoft's technical calculation software Mathcad to solve 100 diverse problems in electrostatics and electromagnetic wave theory, and may be of interest to students, educators and engineering professionals. Topics covered include analysis of charge and current-carrying systems; vector operators such as divergence, curl and Laplacian; Coulomb's and Gauss' laws; electric and magnetic potential; currents and conductors; capacitance and dielectric materials; inductance and ferromagnetic materials; magnetic circuits; and waveguides and transmission lines.

■ Spectroscopy

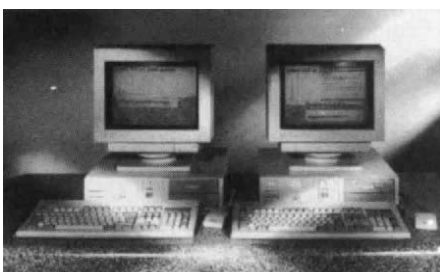
UniQuant is a univariate **quantitative analysis program for spectroscopic data** available from Galactic Industries (*Reader Service No. 112*). This add-on application uses spectral band height/band area measurements to perform univariate quantitative analysis. The program allows the user interactively to select and view spectral bands for quantitation. Band selection options include single or multiple point baseline correction, extrapolated baselines, basepoint averaging, band height, band area and band maxima. Multiple components may be analysed and a reference band for thickness correction can also be included. Calibration choices include reporting raw values, single point calibration or multipoint calibration. The program's user interface automatically displays and updates statistics and the calibration curve as the user adds calibration samples or changes the parameters of the fit. UniQuant runs under GRAMS/386 or derived products version 2.0 or higher on 386 and 486 PCs and compatible computers.

A new hypermedia-based program providing support in the analysis of molecular spectra is now available from Chemical Concepts. As an **electronic reference and analysis tool for molecular spectroscopy**, the company says that SpecTool contains spectroscopic data on all the most important classes of substances in organic chemistry — spectra, structure or reference data (*Reader Service No. 113*). The characteristic features of the ^1H -NMR, ^{13}C -NMR, IR, MS and UV/Visible spectra are given for each class. SpecTool offers a wide range of useful ta-

bles and calculation modules for spectrum analysis. Examples include tools for estimating NMR chemical shifts and coupling constants, tables of isotope abundances and a tool for predicting fragmentation patterns in MS, prediction of C-O stretching frequencies in noncyclic alcohols for IR. The Macintosh version is available now; a version for Microsoft Windows will be shipping in the next few months.

■ Chromatography

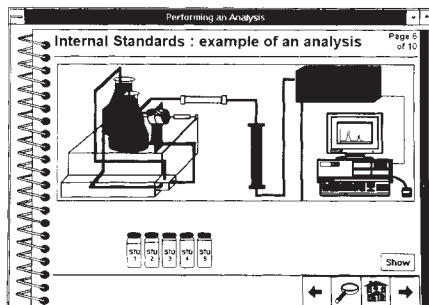
XChrom, Fisons Instruments' V/G Data Systems' **chromatography data system**, is now available on the Microsoft Windows NT operating system (*Reader Service No. 114*).



XChrom now available for Windows NT.

Designed for both networks and Windows, XChrom offers graphical data handling, icons, tool bars, file services and context-sensitive help. The company says that Windows NT allows the PC user access to operating system capabilities usually associated with large, multiuser systems. Security features and multiple processing capabilities have been built in.

The new **HPLC training program** from HPLC Technology is designed to teach the rudiments of HPLC theory and practice



HPLC: the theory and the practice.

(*Reader Service No. 115*). The package is divided into five sections: chromatographic theory, HPLC instrumentation, performing an HPLC analysis, alternative LC methods, and questions and answers on HPLC.

■ Earth sciences

DIMPLE is a new interactive **image processing system for earth scientists** from Cherwell Scientific that provides researchers with tools for analysing remotely sensed images (*Reader Service No. 116*). It brings an array of multispectral analysis and classification techniques to the Macintosh plat-

form, in addition to providing a full range of image analysis, enhancement, transformation and registration tools. The Image Operation Language allows users to tailor the package to suit individual needs. The UK£1,500 program supports vector data and 24-bit images, and allows the user to work with large images with up to 32,000 channels, either from disk or in memory.

Version 4.1 of Earth Resource Mapping's **image processing software for remote sensing** and digital mapping applications is now shipping (*Reader Service No. 117*). E R Mapper 4.1 will be released on CD-ROM for a variety of workstation platforms — Sun, Digital and Silicon Graphics. Enhancements include: all ER Mapper licences float to any supported platform; faster optimized image rectification and geocoding; sample SAR datasets and processing algorithms, direct reading of ARC/INFO vector coverages; and enhanced display of 24-bit map composition overlays on 8-bit displays. □

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Career management for scientists

In the first of a monthly series of articles, written in the main by people who are professionally involved in recruitment, Larry Botheras sets out some ground rules for those considering a career move into industry.

THE management of their careers is probably something that sits close to the bottom of most people's lists of priorities while the job is going well. Unfortunately, it is most needed at times of 'distress', for example when funding is about to run out, or cuts are being imposed. Considering the way in which most careers start off, and the generally uninformed way in which one makes life-directing decisions at a young age, perhaps there is a much greater scope for proactivity.

To expand on the problems: typically, academic careers are initiated during school years, when, frequently, advice on careers is given by a teacher who has probably not spent any time out of an academic environment. This undoubtedly colours the advice given (the principle being to recommend what you know best) and it is further reinforced by any advice — both formal and informal — given at university level, which tends to be on the same basis. Unfortunately, for most people there is a time in their lives at which they find themselves bored with their role, or thinking that they have made the wrong choice and want a change. (For scientists, this is typically reflected in the desire to get away from the bench.) It is often the time when recruiters, such as myself, become involved in helping to pick up the pieces. This can be an extremely traumatic time for any individual, who may have little idea of how a portfolio of skills may be used in an alternative career.

The pharmaceutical and health care industry can be a highly beneficial provider of alternatives for the life science population, with a wide range of opportunities through which careers can evolve away from the bench. Typical routes for scientists moving into industry are clinical trials, regulatory affairs, project management, medical information or strategic product development. These areas are of critical importance to companies and call for a good scientific training, giving the new initiate 'street credibility'. Subsequent development will depend on the ability of the individual to interact well with a wide range of people with divergent interests.

These disciplines can be summarized as follows:

● **Clinical trials** — the management of studies carried out in man on new products. Typically this involves liaison with physicians and nurses at investigating centres (hospitals primarily), collection of the

data and coordination of the trial.

● **Regulatory affairs** — liaison with governmental organizations to acquire a drug licence. This involves considerable contact with the clinical research team to ensure that the trial is likely to produce data of an acceptable standard.

● **Project management** — the overall management of a drug development programme, including timing, finance and resources. It will involve liaison with groups from the research, development, clinical, regulatory affairs, manufacturing and marketing areas.

● **Medical information** — answering written and verbal queries from both healthcare professionals (doctors, pharmacists) and the general public. It also includes maintenance of up-to-the-minute information on the competition as well as one's own products, including adverse reactions.

● **Strategic product development** — the assessment of new ideas, products and therapeutic areas to develop company R&D strategy for the future. It involves working with marketing as well as senior management and R&D.

There is, of course, a fairly major distrust of industry by those in academic institutions, in my view fuelled primarily by ignorance. The standard view of pharmaceutical research has been that options are limited, pressure is high and the drive for profitability is relentless. Certainly, research is more directed, but, in the end, very little research anywhere is done on an altruistic basis — everyone has to generate funding, and that always means project justification. It is a fact that a considerable amount of original medical science has been carried out by the pharmaceutical and healthcare industries, with a number of the most capable individuals at funded institutes winning Nobel prizes.

Job hunting

So you are persuaded — but what are the issues to be addressed in looking for a job in industry?

● **Presentation** — The first major area for improvement is usually the written career history. If applying for a research job in industry this is less applicable, but for other positions a serious rethink is important. The main criticism that I have is the tendency for academic CVs to be judged on thickness. The package needs, on the contrary, to be succinct, highlighting specific achievements rather than rambling

through many details. Certainly include a list of publications, but if it is long, abridge it to include the most recent or most relevant. The recruiter, if interested, can then ask for a full list.

Second, try to put yourself in the position of the recruiter. He may have 200 CVs to look at, so try to stand out. That means focusing the application on the particular opportunity and ensuring that it looks as if time has been taken with the presentation.

● **Where to apply** — The advice here is relatively simple: start networking among colleagues and contacts, among whom there are bound to be people who have moved into the industry. Ask them to whom you could talk. The important thing about networking is not to put contacts under pressure. Do not ask them if they know of a vacancy — this puts them under pressure in two respects; through the possibility of letting you down, or through their reputation being put at risk within their company by recommending you.

Obviously, you must assiduously scan the appointments pages of journals. The obvious international journal is *Nature*, but there are also specific industry journals, the best known of which is *Scrip* — published twice weekly. It is highly specialized, but it is a good place to see some relevant advertisements.

Finally, there are a number of specialist recruitment companies that concentrate on recruiting for the industry, so use your network contacts to find the most appropriate for your interests. Send a copy of your career history and wait for the response.

Final thoughts

Once you have started the networking process, do not give up on it the minute you find a job. Your network in its new, extended form is a valuable asset and needs to be nurtured. You will possibly need to use it on other occasions.

Over the past few years there has been a move away from the 'career for life' syndrome, so keep your mind open as far as possible. Remember that there is only a small gap between preconceptions and prejudices.

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